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THERMODYNAMIC ASPECTS OF A PROPOSED MECHANISM FOR ACETATE OXIDATION IN BACTERIA

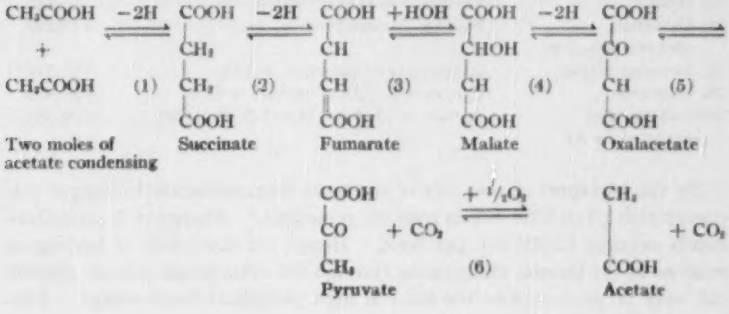
By Benjamin V. Siegel

Department of Bacteriology and Experimental Pathology, Stanford University School of Medicine, Stanford, California

Communicated by V. C. Twitty, July 10, 1950

While thermodynamics does not pretend to postulate the mechanism for a given process, the details of which actually fall within the realm of kinetics, it does purport to predict the possible feasibility of such a mechanism. With a knowledge of the difference in energy and entropy between the starting substance and each of the intermediates involved, the question as to how far a given reaction may go can be answered with confidence without complicated experimentation. In this note the rationale of chemical thermodynamics has been applied to a mechanism recently proposed for the oxidation of acetate by *Escherichia coli*.

Ajl,[1] employing inhibition experiments with arsenious oxide and cyclohexanol as inhibiting agents and a modified version of the Stanier[2] technique of simultaneous adaptation, has obtained evidence against the occurrence of the Krebs oxidation cycle in the respiration of *E. coli* and *Aerobacter aerogenes*. From his findings he has been led to suggest the following series of steps in the oxidation of acetate by *E. coli*.



The free energy changes for each of these reactions can be calculated from the free energies involved in the formation of the respective compounds concerned, and these summed up to give the free energy change for the over-all reaction. Such calculations have been made using the values listed in table 1.

The free energy changes for the various steps of this system are given in table 2. Combination of the six steps gives an over-all balance of −51,385 cal., that is, the complete system is exergonic by a substantial margin.

TABLE 1

FREE ENERGIES OF FORMATION OF MOLECULES INVOLVED IN ACETATE OXIDATION BY *E. coli*

SUBSTANCE	$-\Delta F_f^\circ$ CAL. MOLE^{-1}
O_2 (g, 0.2 atm.)	950
CO_2 (g, 0.0003 atm.)	99,060
H_2O (l)	56,560[a]
Ions	. . . . .
Acetate	89,700[a]
Succinate	166,360
Fumarate	142,525[b]
Malate	199,430[b]
Oxalacetate	184,210[b]
Pyruvate	106,460[b]

[a] Values calculated unless otherwise indicated.

TABLE 2

ACETATE OXIDATION BY *E. coli*

STEP	REACTION	ΔF, CAL.
(1) Condensation and dehydrogenation	Acetate + acetate $\rightleftharpoons$ succinate	+13,040
(2) Oxidation (dehydrogenation)	Succinate $\rightleftharpoons$ fumarate	+23,835
(3) Hydration	Fumarate + $H_2O \rightleftharpoons$ malate	−345
(4) Oxidation (dehydrogenation)	Malate $\rightleftharpoons$ oxalacetate	+15,220
(5) Decarboxylation	Oxalacetate $\rightleftharpoons$ pyruvate + CO_2	−21,310
(6) Oxidation	Pyruvate + $\frac{1}{2}O_2 \rightleftharpoons$ acetate + CO_2	−81,825
Over-all reaction postulated by Ajl	Acetate + H_2O + $\frac{1}{2}O_2 \rightleftharpoons 2CO_2$ + 6H	−51,385

By the transport of one pair of electrons from substrate to oxygen 3+ energy-rich phosphate bonds may be generated.[6] Energy-rich phosphate bonds average 12,000 cal. per bond. Hence, for the 3 pair of hydrogens evolved in the process and passing through the cytochrome system 108,000 cal. may be generated in the form of high phosphate bond energy. This

together with the 51,385 cal. resulting from the free energy changes in the over-all reaction gives a total energy of 159,385 cal. available for useful work.

The free energy change for the complete oxidation of acetate to carbon dioxide and water is −214,840 cal. However, Clifton and Logan[7] and Siegel and Clifton (experiments to be published) have demonstrated that *E. coli* oxidizes acetate to only three-fourths completion. These workers, employing manometric methods, found that with acetate as the sole substrate in a pH 7.2 phosphate buffer medium, washed cell suspensions carried out the oxidation to 74.9 per cent completion, and with a resultant R. Q. of 0.96. This suggested the stoichiometric equation for oxidative assimilation as being:

$$2C_2H_4O_2 + 3O_2 \rightleftharpoons (CH_2O) + 3CO_2 + 3H_2O.$$

As a first approximation, the free energy change for the oxidative assimilation process was calculated to be −160,915 cal. (−214,840 × 0.749). This, according to thermodynamic reasoning, would be the energy actually available to the cell for the performance of useful work. As noted above, calculations made in line with the proposed mechanism for the acetate oxidation give a ΔF value of −159,385 cal. These two values are then in very close agreement, which might suggest that the process as proposed could be the correct one.

In any case, the mechanism for acetate oxidation by *E. coli* as intimated by Ajl's recent findings seems to have a thoroughly sound basis in thermodynamic formulations.

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THE THICKNESSES OF HEMOGLOBIN AND BOVINE SERUM ALBUMIN MOLECULES AS UNIMOLECULAR LAYERS ADSORBED ONTO FILMS OF BARIUM STEARATE*

BY ALBERT A. FISK†

GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA 4, CALIFORNIA‡

Communicated by Linus Pauling, July 17, 1950

The following work, which describes a method of measuring one dimension of some protein molecules, is based on the determination of the apparent thickness of a unimolecular layer of globular protein molecules adsorbed from solution onto a metallic slide covered with an optical gauge of barium stearate. Langmuir[1, 2] and Rothen[3] have published a few results obtained by such a technique, but have not exploited the method thoroughly. A complete set of experimental data has been obtained by Clowes[4] on insulin and protamine. He studied the effects of *p*H and time of exposure on the thickness of layers of protamine and insulin adsorbed onto slides covered with barium stearate and conditioned with uranyl acetate. He found that the *p*H was responsible for large variations in the thickness of the adsorbed layers and that the thickness of insulin layers adsorbed onto a protamine base was dependent on the concentration of the insulin. Since Clowes found thicknesses as high as 100 Å. for protamine and 400 Å. for insulin, he was without doubt usually dealing with multilayers.

Experimental.—The apparent thickness of a protein layer is measured with an optical instrument called the ellipsometer by Rothen,[5–7] who has given a complete description of its design and optics and has calculated its sensitivity as 0.3 Å. This instrument measures the ellipticity of light reflected from a metallic slide when it is covered with a thin film of transparent material. The parameters of the ellipse of polarization are determined in part by the thickness and refractive index of the transparent layer of material on the slide. The ellipsometer, which is a type of polarimeter, uses the half-shadow technique, by which a change in the ellipticity of the reflected light requires a change in angular setting of an analyzer used to balance the intensity of the half fields. As actually used, the instrument is calibrated with films of barium stearate, which for different known thicknesses, previously determined by x-ray diffraction measurements, require different angular settings of the analyzer for equal intensity of the half fields. In this way one measures the angular change in the analyzer setting produced by a film of unknown thickness and relates it by a calibration curve to a known thickness of barium stearate.

Such a calibration assumes that the indices of refraction of films of

barium stearate and globular proteins are the same. The indices of refraction of hemoglobins and serum albumins in the unhydrated state have been calculated by Putzeys and Brosteaux[8] and by Armstrong[9] to be 1.60. Using data obtained by Bull[10] on the hydration of proteins in equilibrium with various partial pressures of water, one can correct the refractive index of 1.60 for hydration of the dry films resulting from the relative humidity of the laboratory. By using the Lorentz-Lorenz equation for such a mixture of protein and water, one obtains an effective refractive index of 1.57. However, it is reasonable to suppose that the globular protein molecules do not occupy the entire surface of the barium stearate, but pack together in such a way as to leave voids between themselves. This condition will further reduce the refractive index, which will be dependent on the type of packing assumed for the adsorbed protein molecules. It has been assumed that when the barium stearate surface is saturated with a monolayer of protein molecules the packing may be best approximated by the closest packing of elliptical cylinders resting on their bases. The fractional volume occupied by such a molecular model is 0.91, and if one assumes that the voids are occupied by air the effective refractive index, calculated by the Lorentz-Lorenz equation for a mixture, is 1.50. Since the refractive index of a barium stearate film is 1.50, the apparent thickness of a film of globular protein molecules, if one makes the above assumptions, is optically equivalent to the thickness of a film of barium stearate. Other assumptions concerning the degree of hydration and the percentage of voids would change the apparent measured thickness of the protein molecules by 1 or 2 Å.

The general technique used in these experiments was similar to that used by Rothen[3] in his work on antigen-antibody reactions. With the Blodgett and Langmuir[11] methods an optical gauge of barium stearate was placed on highly polished stainless steel slides which had been thoroughly cleaned with Shamva, a metallographic polish. The stearic acid, dissolved in redistilled benzene, was spread on redistilled water. The zero point reading was then determined on the ellipsometer. The carbonmonoxyhemoglobin solutions were prepared from crystalline carbonmonoxyhemoglobin preparations obtained by the method of Drabkin,[12] and all dilutions were done with 0.003 *M* potassium phosphate buffer at *p*H 6.8. Concentrations of carbonmonoxyhemoglobin were determined colorimetrically on a Klett colorimeter, which had been calibrated by Kjeldahl analyses for nitrogen. The crystalline bovine serum albumin, obtained from Armour's Research Laboratories, was dissolved in acetate buffer of ionic strength 0.15 and *p*H 4.9.

For the adsorption it was found that the most reproducible results were obtained by placing the slides covered with barium stearate for five minutes in 5-ml. beakers containing the protein solutions. The slides were then

thoroughly washed in running distilled water for five minutes. For constant results a thorough and reproducible washing procedure was found to be of importance. After washing, the slides were allowed to dry in air, and the thicknesses of the adsorbed protein layers were then measured on the ellipsometer. All solutions and wash water were maintained between 16 and 19°C. The error for any one set of measurements was about 10%.

Results.—The results may be seen in Table 1 and Figure 1.

TABLE 1

THE APPARENT THICKNESSES OF SOME PROTEIN FILMS AT VARIOUS CONCENTRATIONS OF PROTEIN

CONCENTRATION, G./100 ML.	HUMAN CARBON-MONOXYHEMOGLOBIN, d IN Å.	HORSE CARBON-MONOXYHEMOGLOBIN, d IN Å.	BOVINE SERUM ALBUMIN, d IN Å.
5	37	..	35
4	39	..	..
3	38	37	34
2	39	34	..
1	37	34	32
0.8	35	40	..
0.6	32	36	..
0.5	..	..	27
0.4	37	32	..
0.2	30	29	..
0.1	25	36	25
4×10^{-3}	19	..	..
2×10^{-3}	..	39	23
8×10^{-4}	12	..	..
1.6×10^{-3}	13	..	..
8×10^{-4}	..	33	..
2×10^{-4}	..	..	10
8×10^{-5}	..	35	..
5.3×10^{-5}	..	25	..
2×10^{-5}	..	..	0
5.3×10^{-5}	..	25	..

Interpretation of Results.—The data in Table 1 have been interpreted by using the simple Langmuir adsorption equation. If one assumes that the apparent thickness of the film, d, is proportional to the fraction of the surface covered, one obtains as the equation for the adsorption of a unimolecular layer of protein molecules onto a solid surface

$$C/d = 1/bd_m + C/d_m,$$

where

C = concentration of protein in g./100 ml.,
d = apparent thickness of the film,

d_m = the apparent thickness of a unimolecular layer of close-packed protein molecules, and

b = a constant related to the heat of adsorption.

It is seen that a plot of C/d against C should give a straight line, and that the reciprocal of the slope of this line is the apparent thickness of a unimolecular layer of protein molecules. Figure 1 shows that a straight line was obtained, and the reciprocals of the slopes of these lines give the thickness of the horse carbonmonoxyhemoglobin molecule as 36 Å., the human carbonmonoxyhemoglobin molecule as 38 Å., and the bovine serum albumin molecule as 34 Å.

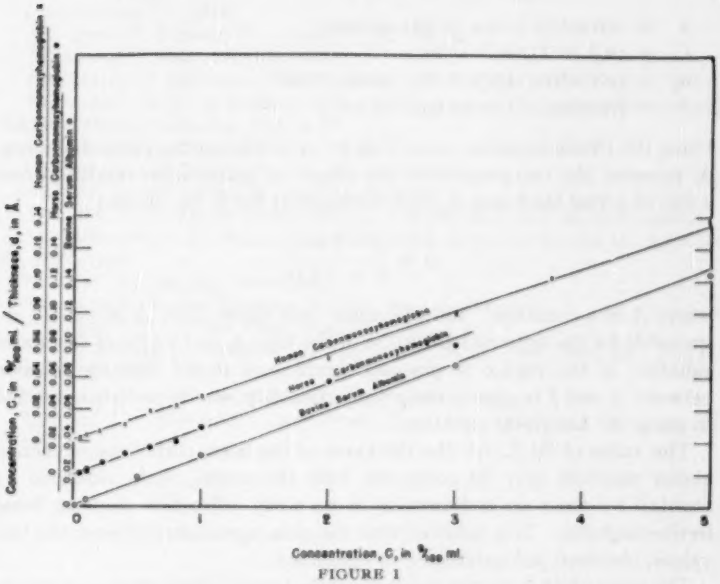

FIGURE 1

Langmuir adsorption isotherms for proteins adsorbed onto barium stearate, t = 16–19°C.

In one respect the conditions of our experiments departed from those assumed in the derivation of the adsorption isotherm equation; namely, that an equilibrium does exist between the molecules in solution and the adsorbed molecules. Our washing procedure amounted to placing the slide in an infinitely dilute solution, and one would therefore expect that, given enough time, all molecules would be desorbed. Our results indicate

that the five-minute period of washing was sufficient to remove any multilayers of hemoglobin adsorbed onto the initial layer, but did not appreciably affect the number of molecules adsorbed onto the barium stearate. That the rate of desorption can be very slow is seen in the horse carbonmonoxyhemoglobin experiments, where concentrations of 8×10^{-6} g./100 ml. or 10^{-3} molar apparently still gave unimolecular layers.

A second factor to consider is the hypothesis that the apparent thickness of the film, d, is proportional to the fraction of the surface covered. The Lorentz-Lorenz equation for a mixture of protein and air may be written

$$(n^2 - 1)/(n^2 + 2) = fC_s,$$

where

n = refractive index of the mixture,
$C_s = (n_p^2 - 1)/(n_p^2 + 2)$,
n_p = refractive index of the protein, and
f = fraction of the surface covered.

Using the Drude equation, $\Delta = -A(1 - 1/n^2)d_m$, for the phase difference, Δ, between the components of the ellipse of polarization resulting from a film of actual thickness, d_m, and substituting for n, one obtains

$$\Delta = \frac{-3Ad_m}{2 + 1/(fC_s)},$$

where A is a constant. Since Rothen[7] has found that Δ is mainly responsible for the apparent thickness of the film, d, and a plot of the above equation in the region of physical significance shows that the relation between Δ and f is approximate proportionality, one is probably justified in using the Langmuir equation.

The value of 36 Å. for the thickness of the horse carbonmonoxyhemoglobin molecule may be compared with the value, 34 Å., obtained by Perutz[13] for the $c \sin \beta$ dimension in his x-ray diffraction work on horse ferrihemoglobin. It is believed that the close agreement between the two values, obtained independently, is significant.

The value of 34 Å. for the thickness of the bovine serum albumin molecule is to be compared with the value 40 Å. resulting from measurements on the double refraction of flow of bovine serum albumin made by Edsall and Foster[14] and with the value 38 Å. for the human serum albumin molecule obtained by Oncley, Scatchard and Brown.[15] Since the 40 Å. and 38 Å. values are for the minor axes of prolate ellipsoids of revolution, one would expect our value of 34 Å., which is an average thickness value, to be less.

The unimolecular layer method of determining one dimension of a globular protein molecule can presumably be applied to other proteins. In conjunction with the other methods of measurement it should prove

helpful in providing information about the size and shape of protein molecules.

The author is very grateful to Drs. Linus Pauling, Dan Campbell and John Singer for their interest and many helpful suggestions.

Summary.—By use of an optical method it has been found that the thickness of a unimolecular layer of human carbonmonoxyhemoglobin molecules adsorbed onto a film of barium stearate is 38 Å., that of horse carbonmonoxyhemoglobin molecules is 36 Å., and that of bovine serum albumin molecules is 34 Å.

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† American Cancer Society Fellow. Present address: Biochemical Laboratory, University of Virginia, University Station, Charlottesville, Virginia.

‡ Contribution No. 1422.

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SEXUAL HORMONES IN ACHLYA. VI. THE HORMONES OF THE A-COMPLEX*

By John R. Raper

Department of Botany, University of Chicago

Communicated by R. E. Cleland, July 1, 1950

The mechanism of hormonal control of sexual processes in heterothallic species of Achlya comprises a number of successive stages, each exhibiting complete dependence on that immediately preceding it.[1] Four morphological phases, each initiated and controlled by one or more specific hormones, are recognized: (1) the production of antheridial hyphae on the ♂ plant in response to the hormones of the *A-Complex* secreted by the vegetative ♂ and ♀ plants; (2) the production of oögonial initials on the ♀ plant in response to hormone *B* secreted by the antheridial hyphae; (3) the chemotropic attraction of antheridial hyphae to oögonial initials and the differentiation of antheridia in response to hormone *C* secreted by the oögonial initials; and (4) the delimitation of oögonia and the differentiation of the oögonial contents to form oöspheres in response to hormone *D* secreted by differentiated antheridia. The dual roles of hormones *C* and *D* indicate the probability that each is a hormonal complex consisting of two or more specific substances. Thus the interchange between the two sexes throughout the entire sexual reproductive process consists of specific, diffusible substances, with the exception of the physical transfer of ♂ nuclei in fertilization.

It was recognized early in the work that the ultimate understanding of the correlative mechanism must depend upon the stepwise elucidation of the successive stages, with concurrent development of the ability to control exactly those stages in the chain of events prior to that of immediate interest. A large part of the work has, therefore, been concentrated upon the initiation of the entire sexual progression (the production of antheridial hyphae) and the factors which quantitatively affect this response.[2, 3] Continued work has furnished more exact detail concerning the activities of the hormones previously described and has revealed that two new hormones, one secreted by the vegetative ♀, the other by the vegetative ♂, are involved in the control of antheridial hyphal production. The activities of these two new hormones, particularly in combination with those previously described, necessitate a revision of the description of that portion of the hormonal mechanism pertaining to the production of antheridial hyphae.

The work reported here has been done with new isolates of ♂ and ♀ strains of *Achlya bisexualis* and *A. ambisexualis*.[4] All major results of the work prior to 1942 have been confirmed with these new isolates and it is thereby known that they are comparable with the isolates originally

employed. The testing methods for hormones affecting the production of antheridial hyphae have been described in detail elsewhere.[2, 3] The hormone concentrates which have been used include: hormone *A* "standard" solution in acetone containing 5×10^5 U./ml., prepared and standardized in 1948 from partially purified and highly concentrated material from filtrates of *A. bisexualis* ♀ in 1943, and acetone-soluble and water-soluble (acetone-insoluble) fractions of filtrates from ♂ and ♀ strains of both heterothallic species. These latter fractions were prepared from each of the filtrates by the following procedure: diatomaceous earth, "Celite," was added to the filtrate and the water was removed from the slurry by distillation *in vacuo;* the dried material, deposited on the inert filler, was then exhaustively extracted with acetone in a Soxhlet's extraction apparatus to provide the acetone-soluble fraction; and the acetone-insoluble material on the filler was recovered by solution in water.

The initiation of the formation of antheridial hyphae on the ♂ plant occurs within an hour after the introduction of the test plants into water containing the appropriate hormones in the proper concentrations. By the end of three hours the antheridial hyphae are 100–500 μ in length and may be counted easily. Each vegetative hypha is treated as an individual and the average number of antheridial hyphae/3 mm. hyphal tip is taken as an index of the intensity of the reaction. Twenty to 100 or more individuals are counted for each test, the number depending upon the degree of accuracy required for the immediate purpose.

Four different hormones, collectively designated the *A-Complex*, are now known to affect quantitatively the production of antheridial hyphae. Two hormones, *A* and A^2, are secreted by the vegetative ♀ mycelium and each alone is capable of inducing the response. Two other hormones, A^1 (previously designated A')[2] and A^3, are secreted by the vegetative ♂ mycelium and neither can initiate the reaction. Since hormones *A* and A^3 are readily soluble in acetone or dioxane while hormones A^1 and A^2 are not, quantitative separation of the various hormones is readily accomplished.

The different hormones of the *A-Complex* will be considered in the order of their discovery.

Hormone *A*, secreted by the ♀, induces its characteristic response, the production of antheridial hyphae, with an intensity which is a logarithmic function of its concentration (the curve at the lower left of Fig. 1). This relationship obtains over a concentration range from 0.05 to 5000 U./ml., a factor of 100,000. Antheridial hyphae averaging fewer than 30/hyphal tip are most easily and accurately counted. Since maximal responses of this intensity are induced by approximately 50 U./ml., a greatly restricted range of concentrations, 0.5–50 U./ml., is therefore commonly employed.

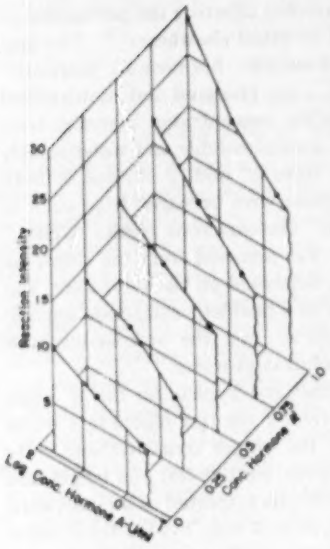

FIGURE 1

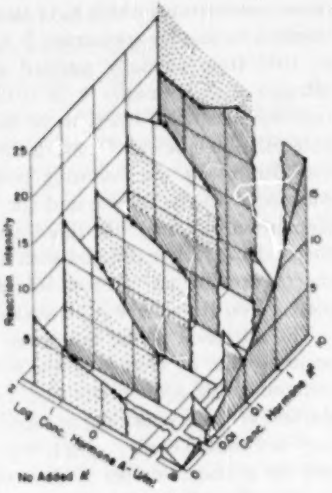

FIGURE 3

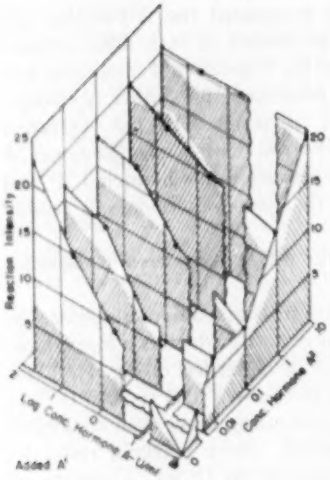

FIGURE 4

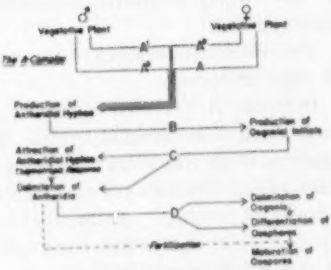

FIGURE 6

See opposite page for description of illustrations.

Hormone A^1, which is secreted by the vegetative ♂ and has no initiating activity of its own, markedly enhances the intensity of the reaction to hormone A. The pattern of effect of A^1 on reaction intensity, at three concentrations of hormone A, is shown by the four upper curves of figure 1, representing four different concentrations of A^1. The responses elicited by hormone A in the absence of added A^1 is shown by the curve at the lower left of figure 1. The data plotted in figure 1 represent the average values for four tests, each point being the average reaction of 80 individual hyphae. From the data given in figure 1 and from the results of other series not included here, it is apparent that for a given concentration of hormone A an optimal concentration of hormone A^1 increases the intensity of the reaction by a factor of about three. For suboptimal concentrations of A^1, concentration of A remaining constant, the reaction intensity is roughly a linear function of A^1 concentration. The concentration range of A^1 over which this relationship holds, however, is very restricted as compared to that of hormone A; the greatest concentration of A^1 used here, that of raw filtrate from a mass culture of ♂ mycelium, approaches an optimal value. Low concentration of either A or A^1 acts to limit the response. An intense production of antheridial hyphae is therefore possible only if both hormones are present in adequate amounts.

No method has yet been devised to determine whether A^1 is indispensable for the production of antheridial hyphae since the only means of testing for the hormone depends upon the reaction of the plant which simultaneously secretes it. Previous work has shown that amounts of A^1

Figure 1. Production of antheridial hyphae on the ♂ plant in relation to graded concentrations of hormones A (♀) and A^1 (♂) when the two hormones are independently varied. In this and in subsequent figures the average number of antheridial hyphae/-3 mm. hyphal tip produced within four hours following the introduction of ♂ plants into the test solutions is used as the index of reaction intensity and the concentrations of hormone A tested were 50, 5 and 0.5 U./ml.

Figure 3. Response, in the absence of added hormone A^1, to graded concentrations of hormone A alone (stippled curve at lower left) and hormone A^2 alone (lined curve at lower right) and to independently varied concentrations of both A and A^2 (four enclosed composite curves). The additive values for specific combinations of A and A^2 are indicated by the superposition of the curve for hormone A alone (stippled) on the projection of the control reaction for A^2 alone (lined) across each curve. Thus the unshaded portion of each curve represents the mutually augmentative effect of the two hormones in combination in excess of the sum of their separate activities.

Figure 4. The effect of added hormone A^1 on the responses induced by hormones A and A^2 in the same concentrations shown in figure 3. The response values for the various A plus A^2 combinations are shown by the shaded portions of each curve. Thus the unshaded portion, if any, of each curve represents the augmentative effect of hormone A^1 on the combined activity of hormone A plus A^2.

Figure 6. Revised diagram of the hormonal mechanism which initiates and coordinates the several phases of the sexual process in heterothallic species of Achlya.

significantly affecting the reaction are secreted by the ♂ plant during the 3–4-hour period required for the test.[2] The quantity of A^1 secreted into the test solution has also been shown to vary rhythmically, each cycle extending over 18–19 hours. Reasonably reproducible reactions may be obtained to a given concentration of hormone A over extended periods, however, if hormone A^1 is added in non-limiting quantity.

Hormone A^2, the third specific secretion involved in the control of the production of antheridial hyphae, is secreted simultaneously with hormone A by the vegetative ♀ and is chemically separable from hormone A. Hormone A^2, like A, induces the production of antheridial hyphae in the absence of other added hormones. The pattern of the response intensity

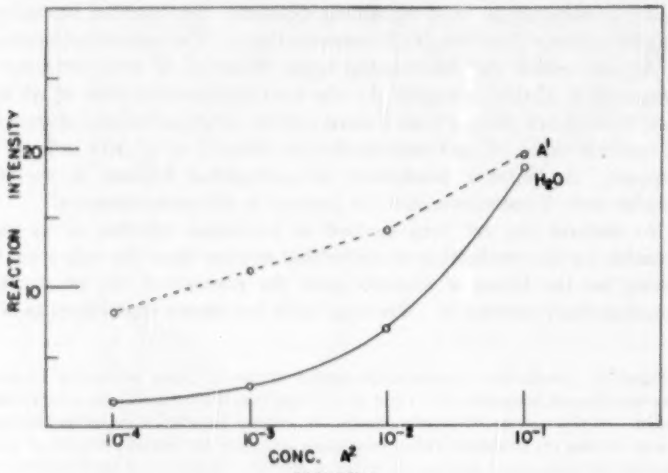

FIGURE 2

Response to graded concentrations of hormone A^2 (♀) in the absence (unbroken curve) and presence (broken curve) of added A^1.

vs. hormonal concentration is quite distinct from that of hormone A, the reaction intensity being roughly an exponential function[3] of the concentration of hormone A^2 (Fig. 2 and lower right curve of Fig. 3). The addition of hormone A^1 augments the response elicited by graded concentrations of hormone A^2 (Fig. 2). The augmentative effect of A^1 on A^2, however, does not resemble that on hormone A. Instead of increasing the response by a constant factor as with hormone A, its effect is most pronounced with low concentrations of A^2 and diminishes with increasing concentration of A^2, having but little effect when the latter is present at ten times its concentration in raw filtrate.

A still different pattern of augmentation results when hormones A and A^2 are combined. A plot of reaction intensity as a function of concentration of hormones A and A^2, when the concentrations of the two hormones are independently varied, is given in figure 3. Each of the two hormones is able, in the absence of the other, to induce the response in the intensities shown by the curves at the lower left and lower right of the figure. The results which obtain in other portions of the plot, i.e., when both A and A^2 are simultaneously present, depend upon both the absolute quantities and the relative concentrations of the two hormones. For example, when A^2 is present in a concentration of 0.01, the response intensity increases markedly with increase in the concentration of A, the reaction in each combination of A and A^2 being higher than the sum of the reactions separately induced by the two hormones. The additive values for all combinations of A and A^2 are indicated in figure 3 by the superposition of the control response curve to hormone A alone (stippled) above the projection of the response to hormone A^2 alone (lined) across the face of each curve. The greater-than-additive effectiveness of the A plus A^2 combinations (the unshaded portions of each curve) is thus seen to obtain in all cases except at the highest concentration of A^2. Actually, the relationship shown in the three central curves of figure 3 probably obtains throughout the concentration ranges of both hormones found in matings of ♂ and ♀ plants. The highest concentration of A^2 used here, 10 ×, would almost certainly never be encountered in a filtrate of ♀ plants or in the liquid in which plants are mated; the concentrations of hormone A used here, however, fall within the range commonly present under mating conditions.

The response induced by combining hormones A and A^2 is further augmented by the addition of an optimal concentration of hormone A^1. The pattern of this augmentation is shown in figure 4, in which reaction intensity (vertical coordinates) is plotted against independently varied concentrations of hormone A and A^2 in the presence of hormone A^1. In this figure the reactions elicited by the specific combinations of A and A^2 in the absence of added A^1 (taken from the data presented in Fig. 3) are shown by the shaded areas on each curve. Thus the augmentative effect of A^1 for the various combinations of A and A^2 is indicated by the unshaded areas on the several curves. The augmentation by hormone A^1 of the response in the various combinations of the hormones A and A^2 is, however, less marked than its effect on either of the ♀-secreted hormones alone and it is greatly reduced in the presence of high concentrations of hormone A^2. Actually, in certain combinations, the augmentative capacity of hormone A^1 would appear to be definitely decreased by hormone A^2, even in low concentrations of the latter. Thus the response to 50 U./ml. of hormone A plus A^1 is reduced from an average number of 22.5 antheridial hyphae/vegetative hypha to 16.1, 17.6, 20.1 and 20.4 by the addition of hormone

A^2 in concentrations of 0.01, 0.1, 1.0 and 10, respectively. Each of these points is based on the counts of 80–200 individual reacting hyphae and the decrease in each case due to hormone A^2 is unquestionably significant. This inhibitory effect is present, however, only at the single highest concentration of hormone A, 50 U./ml.[6]

Hormone A^3, the fourth secretion involved in the quantitative control of the production of antheridial hyphae, has been studied less intensively than the three hormones described above. This is especially true of the complex interactive effects in the various possible combinations with the other hormones. Hormone A^3 is secreted by the vegetative ♂ plant simultaneously with hormone A^1, from which it is easily separable because of its ready solubility in acetone and dioxane, particularly the latter.

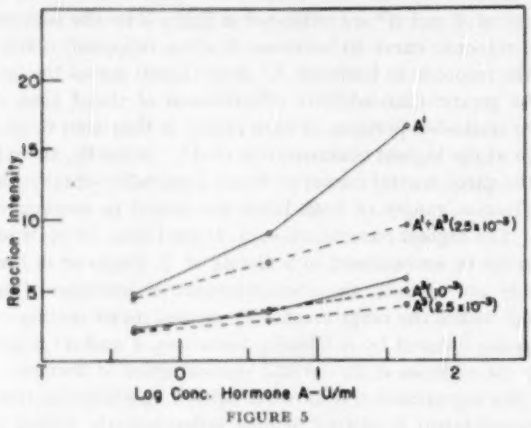

FIGURE 5

Suppression by hormone A^3 of the response induced by graded concentrations of hormone A in the presence and absence of hormone A^1. Solid curves represent control responses; broken curves, the suppressed responses induced in the presence of hormone A^3 (see text for concentrations).

Hormone A^2 acts to limit the number of antheridial hyphae produced by the plant in response to the other hormones of the *A-Complex*. This inhibition has been found to be effective on the response induced by both hormones A and A^3 and, most markedly, on that induced by hormone A in the presence of an optimal concentration of A^1. The pattern of inhibition by hormone A^2 of the response induced by hormone A in the presence and absence of added A^1 is shown by the broken curves of figure 5. The indicated concentrations of A^2 are those of an acetone solution of the hormone derived from the filtrate of mass culture of ♂ mycelium; this solution contained hormone A^2 at 100 times its concentration in the raw

filtrate. The highest concentration used here, one-fourth that of the raw filtrate, significantly reduces the number of antheridial hyphae produced by A alone and by A plus A^1. The slight depression of the reaction by the lower concentration of A^2 at 50 U./ml. hormone A is of doubtful significance.

The effect of hormone A^3 on the reaction induced by A^2 has been tested only in a preliminary series. On the basis of these tests, a slight but significant decrease in the reaction induced by A^2 results from the addition of A^3. No attempts have been made to test the effect of A^3 in more complicated combinations of hormones A, A^1 and A^2.

The chemical identity of none of the hormones of the *A-Complex* is known; furthermore, but little is known about their chemical and physical properties. Certain of the properties of hormone A were described in connection with an unsuccessful attempt to isolate and identify the hormone,[3] but insufficient information was obtained even to assign the category of organic compounds to which the hormone belongs. Hormone A^1 has been found, in preliminary studies, to be readily dialyzable, neutral in reaction, non-migratory in an electric field, quite stable to heat, acid and oxidizing agents, and destroyed by prolonged alkaline hydrolysis; it is readily soluble in water but no other adequate solvent for it has been found. Nothing whatever is known of hormones A^2 and A^3 beyond their solubilities in water and acetone (or dioxane), respectively. Chemical characterization and identification of these hormones of the *A-Complex*, as well as the other hormones effective in the sexual process of Achlya, have been and will continue to be seriously hampered by the relative difficulty of obtaining the raw materials in sufficient quantities.

The four hormones of the *A-Complex* are secreted by vegetative plants in the presence or absence of sexually compatible strains. Furthermore, sufficient interspecific testing has been done to show that the secretions of ♂ and ♀ strains of at least two heterothallic species, *A. bisexualis* and *A. ambisexualis*, are completely comparable and that the A-hormones of either species are completely effective on the ♂ strain of the other species. It does not necessarily follow from this fact that the hormones of the *A-Complex* secreted by the two species are chemically identical.

The titers of three of the four hormones of the *A-Complex* are known to vary characteristically with the age of the cultures in which they are produced. The titer of hormone A, in ♀ cultures, remains low for the first week of vegetative growth after which it increases rapidly to reach a maximum at 10–12 days. Hormone A^1, in ♂ culture, reaches a maximal concentration three to five days after the initiation of vegetative growth and thereafter steadily decreases to approximately one-half its maximal activity at 14 days. The titer of hormone A^2, also in ♂ cultures, builds up to a maximum within the first five days of growth and remains constant

thereafter. No specific study has been made of activity of hormone A^2 (♀) in respect to culture age.

A revised scheme for the mechanism of hormonal control in heterothallic Achlyas, including the two new members of the *A-Complex*, hormones A^2 and A^3, described here for the first time, is given in figure 6. Only the first of the several stages in the sexual reaction, that pertaining to the production of antheridial hyphae, has been subjected to intense quantitative study and it is not unlikely that the hormonal mechanism, as presented here, will require many further revisions as subsequent stages are successively brought under intensive examination.

Summary.—Two new hormones, A^2 and A^3, secreted by vegetative mycelia of ♀ and ♂ strains, respectively, have been shown to be involved in the initiation of the sexual reaction in heterothallic species of Achlya. Thus the quantitative control of the production of antheridial hyphae on ♂ plants depends upon four distinct hormones: hormones A and A^2, secreted by the ♀, and hormones A^1 (originally designated A') and A^3, secreted by the ♂. These hormones regulate the production of antheridial hyphae in such a way that the intensity of the reaction is: (1) a logarithmic function of the concentration of A, (2) an exponential function[5] of the concentration of A^2, (3) a linear function of the concentration of A^1 in the presence of A, (4) increased by A^1 in the presence of A^2 (the precise pattern of augmentation not yet determined), (5) roughly a logarithmic function of the concentration of A^2 in the presence of A or in the combined presence of A plus A^1, and (6) decreased by A^3 in the presence of A or A^2 alone and in the combined presence of A plus A^1. A revised diagram of the entire hormonal mechanism, including the above additions to the *A-Complex*, is given.

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The collection in England and Denmark of materials requisite to this work was made possible by special grants from the Abbott Memorial Fund and the Department of Botany of the University of Chicago. Laboratory facilities and other assistance were most generously provided by Prof. F. T. Brooks of the Botany School, Cambridge University, England, Mr. H. C. Gilson of the Freshwater Biological Laboratory of Ambleside, Westmorland, England, and Prof. Ø. Winge of the Carlsberg Laboratory, Copenhagen, Denmark.

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[2] Raper, John R., *Am. J. Bot.*, **29**, 150 (1942); PROC. NATL. ACAD. SCI., **28**, 509 (1942).

[3] Raper, John R., and Haagan-Smit, A. J., *J. Biol. Chem.*, **143**, 311 (1942).

[4] Collection data, etc., of these materials are given in: Raper, John R., *Bot. Gaz.* (in press). Cultures of ♂ and ♀ strains of *Achyla bisexualis* and *A. ambisexualis*, as well as a number of sexual intergrade strains of the latter species, have been deposited in the Centraalbureau voor Schimmelcultures, Baarn, Holland.

[5] A plot of log log antheridial hyphae vs. log concentration of A^2 reveals a linear relationship. From this it follows that $I = c^{ak}$, where I is reaction intensity, x, the concentration of A^2 and k, a proportionality constant. The author wishes to thank Dr. Leonard J. Savage for pointing out this relationship.

[6] An alternate interpretation of A^2 activity might be that the water-soluble fraction of ♀ filtrate contains A^1 plus an effective contamination of A. The pattern of A^1 augmentation on A^2 response and the expected response curve of an $A + A^1$ mixture, when the two hormones are simultaneously diluted, lend strong support for such an hypothesis. On quantitative grounds, however, it is apparent that no possible mixture of hormones A and A^1 could give either the augmentative or inhibitory effects attributed to hormone A^2 when the water-soluble fraction is added to known concentrations of hormone A in the absence and presence of A^1, respectively (Figs. 3 and 4). It is because of these latter effects that the interpretation presented in this paper is favored over that of a mixture of previously known hormones.

ELECTRON TRANSFER IN INTERMETALLIC COMPOUNDS

BY LINUS PAULING

GATES AND CRELLIN LABORATORIES OF CHEMISTRY,* CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA

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In our discussions of the electronic structure of intermetallic compounds during the last three years brief mention has been made from time to time of the phenomenon of electron transfer.[1-3] The interpretation of the observed interatomic distances in many metallic phases seems to require the assumption that electron transfer has taken place; an example is Al_9Co_2, for which the distances[4] support the charge distribution[5] $Al_9^{-2/9}Co_2^{+1}$. The indication by physical properties of a filled-Brillouin-zone structure for Fe_5Zn_{21} also led to the suggestion of electron transfer:[2] about $^1/_4$ electron is indicated to have been removed from each zinc atom and about one electron added to each iron atom.

In this paper it is pointed out that the analysis of interatomic distances shows that electron transfer takes place in a great many interatomic compounds, and that the numbers of electrons involved are reasonable, in relation to the changes in valence resulting from loss or gain of electrons and to the partial ionic character of the bonds between unlike atoms and the striving of atoms toward electroneutrality.

Let us divide atoms into three classes: hypoelectronic (electron-deficient) atoms, hyperelectronic (electron-excess) atoms, and buffer atoms. Hypoelectronic atoms are atoms that can increase their valence by adding electrons. The hypoelectronic elements include the first three elements of each short period and the first five elements of each long period, as shown in

table 1. Atoms of these elements have more bond orbitals than valence electrons (in the uncharged state), and they can accordingly increase their valence by one unit by accepting an electron. Hyperelectronic atoms are atoms that can increase their valence by giving up an electron. The hyperelectronic elements with respect to metallic compounds include the last three elements (before the noble gases) of each short period and the last seven elements of each long period. Atoms of these elements have more valence electrons than bond orbitals, and they can increase their valence by one unit by giving up one electron of a pair occupying a bond orbital, thus leaving a valence electron in the orbital. Buffer atoms are atoms that can give up or accept an electron without change in valence. The five elements Cr, Mn, Fe, Co, and Ni and their congeners in the other two long periods are buffer elements with respect to metallic compounds;

TABLE 1

CLASSIFICATION OF ATOMS WITH RESPECT TO EFFECT OF CHANGE OF ELECTRON NUMBER ON METALLIC VALENCE

HYPOELECTRONIC ATOMS					ATOMS WITH STABLE VALENCE					HYPERELECTRONIC ATOMS						
	Li	Be	B				C					N	O	F		
	Na	Mg	Al				Si					P	S	Cl		
BUFFER ATOMS																
K	Ca	Sc	Ti	V	Cr[a]	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As	Se	Br
Rb	Sr	Y	Zr	Nb	Mo[a]	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	I
Cs	Ba	La	Ce[b]													
		Lu	Hf	Ta	W[a]	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi	Po	At

[a] These three atoms can accept electrons but not give up electrons without change in valence.

[b] The rare-earth metals may have some buffering power.

they can give up a non-bonding *d* electron or introduce an electron into the incomplete non-bonding *d* subshell without change in metallic valence (Cr, Mo, and W are buffer atoms with respect only to addition of an electron).

Carbon and silicon are placed in a separate class in table 1. Carbon is an element with stable valence, 4; either the addition of an electron to a carbon atom or the removal of an electron from it causes a decrease in its valence. Silicon also has the stable valence 4, except that it may under certain circumstances make use of outer orbitals (3*d*, 4*s*, 4*p*) and achieve some increase in valence through electron transfer. This effect is less important in alloys of silicon than in compounds of the hypoelectronic atoms.

Let us consider the ways in which an intermetallic compound AB might be stabilized by the transfer of an electron from atom B to atom A.

First, an increase in the number of valence bonds and a corresponding increase in stability would result from electron transfer from B to A if A

were hypoelectronic and B were hyperelectronic, or if A were hypoelectronic and B were a buffer, or if A were a buffer and B were hyperelectronic.

Second, according to the principle of electroneutrality[6] an increase in stability would result from a transfer of electrons if it were to result in a decrease in the electric charges on the atoms. Let B be more electronegative than A. The covalent bonds between A and B would then have some ionic character, of such a nature as to give A a positive electric charge and B a negative charge. By transferring an electron from B to A the charges on the atoms can be reduced, and the substance can thus be stabilized. It is very interesting that this effect involves the transfer of electrons to the more electropositive atoms (the stronger metals); that is, in the opposite direction to that of the transfer of electrons that takes place in the formation of ions in electrolytic solutions.

These two stabilizing effects usually operate together, because the electronegativity increases in the sequence hypoelectronic elements, buffers, hyperelectronic elements. Both effects are stronger for compounds of hypoelectronic elements with hyperelectronic elements than for compounds of elements of either of these two classes with buffer elements. Thus we expect electron transfer to be especially important for compounds such as $NaZn_{13}$, less important for compounds such as Al_5Co_2 and Fe_5Zn_{21}, and of little significance for compounds such as Na_2K, FeCr, and Cu_5Zn_8.

In special cases electron transfer may take place even in compounds of two metals in the same class. Stabilizing factors that might operate to this end include the filling of Brillouin zones, the stabilizing of partially filled non-bonding subshells through increase in multiplicity (approach to half-filling) or through completion of the subshell, and the relief of strain resulting from geometric constraints on ratios of interatomic distances through change in bond numbers.

The compound AlP may be taken as a simple example. It has the sphalerite structure, in which each atom is surrounded tetrahedrally by four unlike atoms. Aluminum is a hypoelectronic atom, with normal valence 3 and with single-bond radius 1.248 Å. Phosphorus is a hyperelectronic atom, with normal valence 3 (resulting from occupancy of four orbitals by five electrons) and single-bond radius 1.10 Å. The predicted Al—P distance for valence 3 and bond number n equal to $^3/_4$ is $1.25 + 1.10 - 0.600 \log n = 2.43$ Å. The observed distance 2.35 Å., does not agree with this value, but is exactly equal to the sum of the single-bond radii; that is, the observed distance indicates that each atom forms four single bonds with its neighbors. We are thus led to describe the crystal by saying that it is composed of quadrivalent $\cdot\dot{\mathrm{Al}}^{-}\cdot$ and $\cdot\dot{\mathrm{P}}^{+}\cdot$, with formal charges -1 and $+1$, respectively; and that, because phosphorus is more electronegative than aluminum, each of the four single bonds formed by

each atom has about 25% ionic character, in such direction as to restore the transferred electrons from the aluminum atoms to the phosphorus atoms, achieving at the same time the extra stability that results from ionic-covalent bond resonance.

Comparison of observed and predicted interatomic distances for the thirty other tetrahedral compounds of this type shows that in general electron transfer occurs, with increase in valence of about one unit for each atom.

As another example we may discuss the striking purple alloy Al_2Au, which has the fluorite structure, with $a_0 = 5.99$ Å. Each gold atom has eight aluminum ligates, at 2.59 Å. If gold retained its usual metallic valence, $5^1/_2$, the eight Au—Al bonds would have bond number 11/16, and the corresponding correction $-0.600 \log n = 0.098$, plus the single-bond radii 1.338 for gold and 1.248 for aluminum, would give the predicted Au—Al distance 2.684 Å., which is so much greater than the observed value as to eliminate the assumed valences. Agreement is obtained by assuming gold to have the valence 7; the corresponding radius[3] is 1.303 Å., and the bond-number correction, for $n = 7/8$, is 0.035, leading to 2.586 Å. for Au—Al.

The valence 7 can be achieved by a neutral gold atom (without a metallic orbital). However, in order for gold to have valence 7, aluminum must have valence $3^1/_2$, or greater if significant Al—Al bonds are formed; and hence at least one electron per gold atom must have been transferred to the aluminum atoms. Indeed, the observed Al—Al distance 3.00 Å. for the six aluminum ligates about each aluminum atom corresponds to $n = 0.15$, and indicates that a significant amount of valence of the aluminum atoms is used in these bonds. It is likely that about 1.5 electrons are removed from each gold atom, which would liberate the customary 0.75 metallic orbital, the valence remaining 7; 0.75 electron added to each aluminum atom would increase the aluminum valence to 3.75, of which $3^1/_2$ would be used in bonds to the four gold ligates, and the remainder in Al—Al bonds.

This large amount of electron transfer is not incompatible with the electroneutrality principle. The electronegativity of aluminum is 1.5, and that of gold is 2.5. The difference corresponds to 22% ionic character of the Au—Al bonds, which with valence 7 for gold would lead to the charge -1.54 on the gold atom. To restore it to neutrality 1.54 electrons would have to be transferred to two aluminum atoms.

The proposed structure provides an explanation of the very high melting point (1060°C.) and large heat of formation of the compound.[7] Coffinberry and Hultgren[8] pointed out that the properties of the Al—Au alloys indicate the operation of an unusually strong attraction between aluminum atoms and gold atoms.

As an example of a compound in which electron transfer is relatively unimportant we may discuss $PtSn_2$, which also has the fluorite structure, a_0 being 6.41 Å. The normal metallic valences 6 for platinum and 4 for tin permit the formation of Pt—Sn bonds with $n = 2/3$ and Sn—Sn bonds with $n = 1/6$. The predicted Pt—Sn distance 1.295 + 1.399 + 0.106 = 2.800 Å. is only slightly high, the observed distance being 2.78 Å. The predicted Sn—Sn distance for $n = 1/6$, 3.27 Å., is also slightly higher than the observed value, 3.21 Å., and a small amount of electron transfer is accordingly indicated. The electronegativity values, 2.2 for platinum and 1.7 for tin, lead to about 6% ionic character of the bonds, and to charges on the atoms that would be neutralized by the transfer of 0.36 electron from each platinum atom to two tin atoms. The increase in valence of tin by 0.18 would increase n for the Sn—Sn bonds to 0.20, and would decrease the radius of tin[3] by 0.01 Å.; the predicted distances Pt—Al = 2.79 Å. and Al—Al = 3.20 Å. are in excellent agreement with the observed values.

Electron transfer is especially important in the alloys of the alkali and alkaline-earth metals with hyperelectronic elements and buffer elements. In the formation of many of these alloys from the elements a very large volume contraction is observed, resulting in part from the bond-number correction of interatomic distances due to the increase in valence, and in part from the decrease in single-bond radius of the hypoelectronic atom with increase in valence. Thus, although the normal radius of sodium for ligancy 12, 1.896 Å., is greater than that of lead, 1.746 Å., the replacement of one fourth of the lead atoms in pure lead by sodium atoms, to form the phase $NaPb_3$, leads to a contraction, the bond distance decreasing from 3.492 Å. to 3.446 Å. This decrease is explained by the assumption that electron transfer occurs, with a little less than one electron transferred to the sodium atom. For Na^-, with valence 2, the single-bond radius would be predicted to be 1.439 Å. (0.075 Å. greater than for Mg), which leads to Na—Pb = 3.413 Å., slightly less than the observed value, and the Pb—Pb distance is also predicted to be decreased by about the observed amount, as the result of the increase in valence of lead and in bond number of the Pb—Pb bonds. In many other intermetallic compounds of the alkali and alkaline-earth metals the interatomic distances similarly indicate that electron transfer occurs to such an extent as to increase the valence by about one unit.

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[*] Contribution No. 1454.

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*IMMUNOGENETIC AND BIOCHEMICAL STUDIES OF NEUROSPORA CRASSA: DIFFERENCES IN TYROSINASE ACTIVITY BETWEEN MATING TYPES OF STRAIN 15300 (ALBINO-2)**

BY ALLEN S. FOX AND W. D. GRAY

DEPARTMENT OF ZOOLOGY AND ENTOMOLOGY AND DEPARTMENT OF BOTANY AND PLANT PATHOLOGY, OHIO STATE UNIVERSITY, COLUMBUS, OHIO

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The concept that primary gene action consists of the determination of protein specificities and that genes bear a one-to-one relation to such specificities rests largely on biochemical investigations utilizing Neurospora and immunogenetic investigations of vertebrate erythrocytic antigens.[1, 2] The fruitfulness of this hypothesis has been large, but questions have been raised as to the suitability of the methods and materials used in the above investigations.[3, 4] Accordingly we have recently initiated a series of studies utilizing a new approach.

The material chosen for this work has been the two mating types (*A* and *a*) of Strain 15300 (albino-2), *Neurospora crassa*.[5] Mating type, which may be regarded as a specificity difference rather than a true sexual difference, is determined by two alleles (*A* and *a*) at a locus on the opposite side of the centromere of chromosome I (linkage group *A*) from the locus of albino-2.[6] The object of these studies has been to demonstrate possible differences in antigenic specificity and enzymatic specificity attributable to the mating type locus in addition to the mating type difference.

The results of the antigenic analysis indicate clear-cut antigenic differences between the two mating types.[7] In the course of this work it was necessary to prepare homogenates of whole mycelia. These homogenates were prepared from mycelia of strains 15300*a* and 15300*A* cultured, without shaking, in 100-ml. portions of medium in 250-ml. Erlenmeyer flasks. The medium employed consisted of 5 g. of KH_2PO_4, 7 g. of Difco Yeast Extract and 50 g. of dextrose which were dissolved in distilled water and the volume made to one liter. The sterile medium was inoculated and the cultures were incubated at 30°C. for twelve or thirteen days, after which time the thick mycelial pads were removed by filtration, washed

thoroughly with tap water and then thoroughly with distilled water. The pads were then squeezed to remove as much water as possible, cut into small squares, and lyophilized to complete dryness. The dried mycelium was then reduced in a Waring blendor in 0.85% NaCl buffered at pH 7.4 with 0.005 *M* phosphate (1 g. dried mycelium per 100 ml. saline), and the resultant suspension homogenized in a Potter-Elvehjem homogenizer. The homogenate was dispensed into small tubes, quickly frozen and stored in a deep-freeze until used.

Shortly after the antigenic analysis was initiated it became apparent that there existed a hitherto unreported difference between these two mating types. First, it was noted that when homogenates prepared as described above were allowed to thaw, one darkened rather rapidly at room temperature while the other did not change color. Second, the mycelium (growing on agar slants) of one mating type was observed to darken with age whereas the other did not. In both instances the darkening was due to the production of a brown or black pigment and occurred only in homogenates or old cultures of 15300*a*. While slight darkening of old cultures of 15300 had previously been reported,[8] no difference between the two mating types had been noted. That this difference is not characteristic of our stocks alone was vouchsafed by a similar difference between new cultures of 15300*a* and *A* obtained from Dr. G. W. Beadle. The present paper is a report on the preliminary investigations concerning the nature of this difference.

Throughout this work homogenates were prepared as described above. At the time of use they were allowed to thaw at room temperature, were centrifuged at 6°C. (5000 r. p. m., 25 minutes), and the clear supernatant liquid was pipetted off and used immediately. Optical densities of these supernates were measured by means of a Klett-Summerson photoelectric colorimeter, the blue filter (No. 44) being used for such measurements. During the course of an experiment the supernates, or the mixtures of supernates and test substrates, were incubated at 37.5°C. Test substrates were dissolved in the buffered saline described above. Unless otherwise indicated, the tyrosine solutions used were 0.04 per cent; dopa solutions were made to a concentration of 1 mg./ml. Fresh solutions were made immediately preceding each experiment.

Since the characteristic color of a darkened homogenate was brown or black, it was early suspected that the darkening was due to melanin and that (1) one strain produced a phenol oxidase while the other did not, or (2) both strains produced phenol oxidase but only one (the strain which darkened) synthesized substrate which could be oxidized with resultant melanin formation. In an early experiment pigment development was followed by measuring optical densities (*D*) of supernates of both mating types at intervals, and it was found that the optical density of the supernate

of 15300a increased for about forty minutes and then came to a constant value; a parallel tube of 15300*A* supernate showed no increase in optical activity (Fig. 1). Since phenol oxidase activity was suspected, a 0.04 per cent tyrosine solution was added (1:1) to each of the two supernates

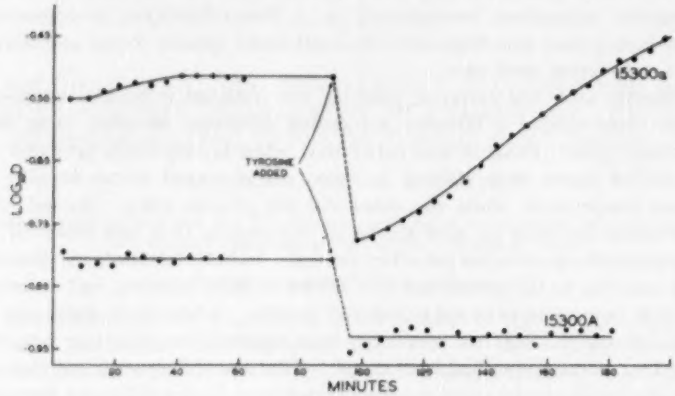

FIGURE 1

Pigment development exhibited by 15,300*A* and 15,300a extracts prior to and following addition of tyrosine.

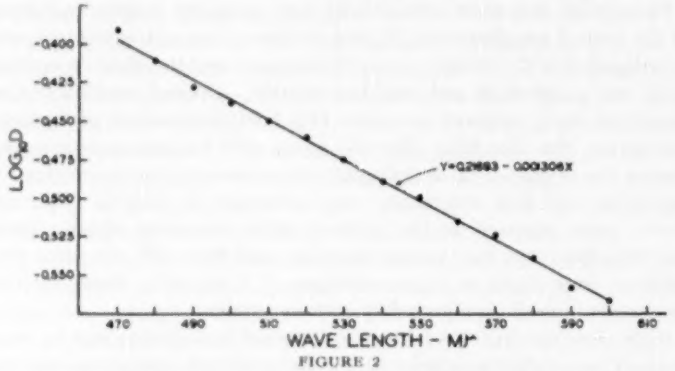

FIGURE 2

Absorption spectrum of pigment developed by 15,300a extracts.

and optical density measurements were continued. With 15300*A* there was no increase in optical density, while with 15300a there was an immediate rise, the magnitude of which is shown in figure 1.

In order to make a more positive identification of the pigment, a tube

of 15300g supernate, to which tyrosine solution had been added, was allowed to incubate until a deep color was developed. Percentage absorption of light of different wave-lengths by this solution was then measured with a Beckman quartz spectrophotometer and an absorption curve

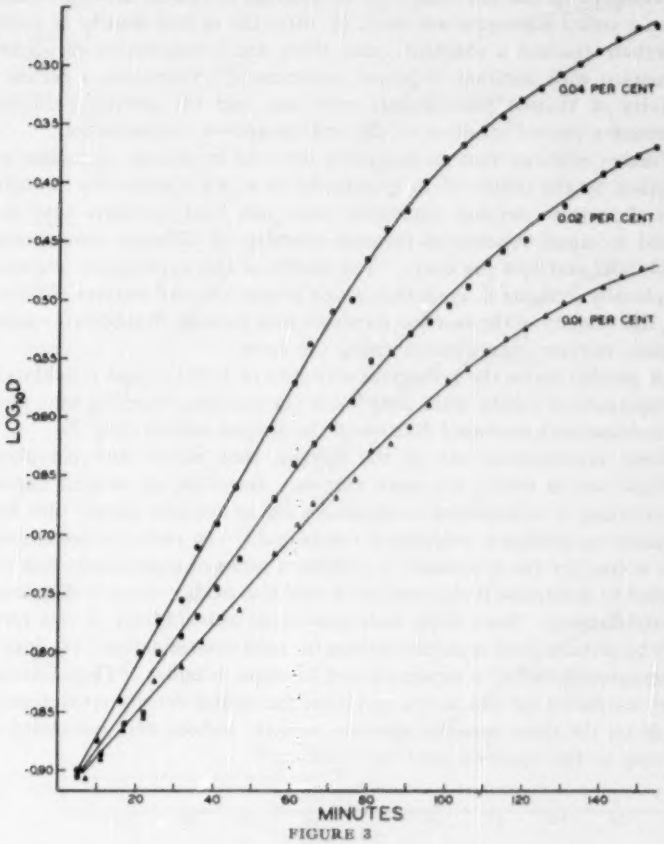

FIGURE 3

Effect of substrate concentration (tyrosine) on activity of 15,300g extracts.

was plotted. This curve, part of which is presented in figure 2, is a straight line between 470 and 600 mμ—a characteristic of the absorption curve of melanin as reported by other investigators.[8, 9, 10] The equation for the straight line fitted to this portion of the curve by the least squares method is $Y = 0.21693 - 0.001306X$, the slope of the curve being closely similar

to that previously reported for melanin. The pigment is furthermore decolorized by potassium permanganate, as is melanin, so that there is little doubt about its identity.

That an enzyme is involved in melanin formation in the present instance is evidenced by the following: (1) no increase in optical density occurred when a boiled supernate was used, (2) after the optical density of 15300a supernate reached a constant value there was a resumption of pigment formation with addition of proper substrate, (3) there was a decline in activity of thawed homogenates with age, and (4) activity of 15300a supernates proved sensitive to pH and electrolyte concentration.

Further evidence that an enzyme is involved in melanin formation was supplied by the results of an experiment in which a supernate of 15300a was allowed to develop maximum color and 5-ml. portions were then added to equal volumes of tyrosine solutions of different concentration (0.01, 0.02 and 0.04 per cent). The results of this experiment, presented graphically in figure 3, show that which is true for most enzyme reactions, i.e., the velocity of the reaction increases with increase in substrate concentration, enzyme concentration being the same.

In another series the tyrosinase activities of 1:2, 1:4 and 1:8 dilutions of supernate of 15300a were tested, and the reaction velocities were found to decrease with increased dilution of the enzyme extract (Fig. 4).

Some investigators are of the opinion that mono- and polyphenol oxidases are in reality the same enzyme; therefore, an enzyme capable of oxidizing a monophenol compound such as tyrosine should also have capacity to oxidize a polyphenol compound.[11] In order to determine if this is true for the tyrosinase of 15300a, a series of experiments was conducted to determine if this enzyme would also oxidize dopa (1-dihydroxyphenylalanine). Since dopa undergoes rapid autoxidation, it was necessary to prepare three separate systems for each determination: (1) dopa + supernate, (2) buffer + supernate and (3) dopa + buffer. These mixtures were incubated for two hours, and from the optical density measurements made on the three separate systems, activity indices were calculated according to the equation used by Ginsburg:[10]

$$A = \frac{D_r - D_m}{D_d},$$

where

D_r = optical density—extract + dopa,
D_m = optical density—extract + buffer,
D_d = optical density—buffer + dopa.

An activity index greater than 1 indicates enzymic oxidation of the substrate, an activity index of 1 indicates no enzyme-catalyzed oxidation,

and an index of less than 1 is indicative of inhibition of substrate oxidation. Results of five experiments are presented in table 1, demonstrating quite clearly the presence of dopa oxidase in 15300*a* but not in 15300*A*. The

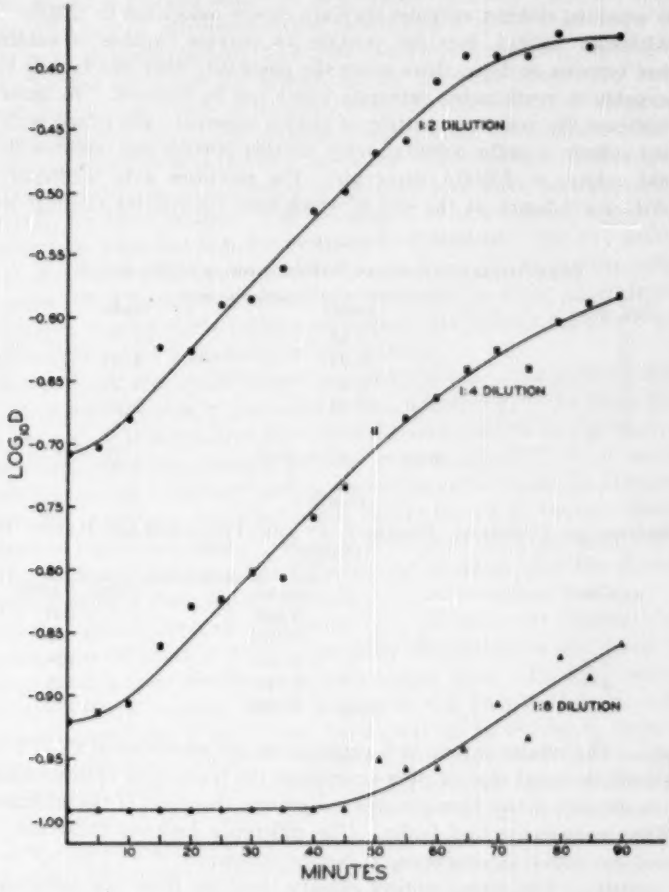

FIGURE 4

Effect of extract dilution on tyrosinase activity of 15,300*a* extracts.

mean activity index of 15300*a* is significantly larger than 1 ($t = 4.8$, $df = 4$, $p < 0.01$), while the mean activity of 15300*A* differs from 1 only with doubtful significance ($t = 2.7$, $df = 4$, $p = 0.05$). The difference

between the two mating types, as tested by paired comparisons, is highly significant ($t = 8.1$, $df = 4$, $p < 0.001$).

While these data do not necessarily prove that monophenol oxidase and polyphenol oxidase are identical, they do demonstrate that if there are two separate, distinct enzymes they are closely associated in 15300*a*.

Although 15300*A* does not contain an enzyme capable of oxidizing either tyrosine or dopa, there exists the possibility that this mating type is capable of synthesizing substrate which can be oxidized. In order to investigate this point one portion of 15300*a* supernate was mixed with an equal volume of buffer solution, while another portion was mixed with an equal volume of 15300*A* supernate. The mixtures were incubated at 37.5°C. for 2 hours, at the end of which time colorimeter readings were

TABLE 1

DOPA OXIDASE ACTIVITY IN HOMOGENATES OF 15300*a* AND *A*

EXPT. NO.	ACTIVITY INDEX	
	15300*A*	15300*a*
1	0.86	1.71
2	0.94	1.80
3	1.00	2.22
4	0.82	1.57
5	0.73	1.31
	Mean = 0.87 ± 0.05	1.72 ± 0.15

TABLE 2

FORMATION OF OXIDIZABLE SUBSTRATE BY THE TYROSINASELESS MATING TYPE (15300*A*)

EXPT. NO.	INCREASE IN OPTICAL DENSITY	
	CONTROL	15300*A* + 15300*a*
1	0.082	0.098
2	0.068	0.084
3	0.094	0.108
4	0.094	0.118
	Mean = 0.0845	0.102

made. The results of four such experiments are presented in table 2 and it should be noted that in each experiment the increase in optical density of the mixture of two homogenates was greater than that of the mixture of 15300*a* homogenate and buffer. The difference between these two, as tested by paired comparisons, is highly significant ($t = 7.95$, $df = 3$, $p < 0.01$). The higher optical density resulting from the addition of inactive 15300*A* supernate to 15300*a* supernate indicates the presence of suitable substrate in the former. Thus, 15300*A* synthesizes substrate for which it is incapable of synthesizing an oxidizing enzyme.

These latter data also make it improbable that 15300*A* supernates fail to exhibit tyrosinase activity because of the presence of an inhibitor rather than the absence of an enzyme, as in the case for the pantothenicless

mutant.[12] The addition of 15300*A* supernates to 15300*a* supernates has no apparent inhibitory effect on the latter.

A final observation seems important. Seitz filtration of 15300*a* supernates results in marked reduction of tyrosinase activity. This reduction of activity can be attributed either to adsorption of the enzyme on the filter or to an association of the enzyme with small particles, not thrown down by the centrifuge but filtered out by the Seitz filter. The latter possibility is particularly attractive, since the association of tyrosinase activity with submicroscopic particles has previously been demonstrated.[13]

Discussion.—The presence of a tyrosinase in 15300*a* and its absence in 15300*A* constitutes a difference in protein specificity not previously observed for these strains. The association of this difference with the mating-type locus has not, however, been established. The two mating types of 15300 are not known to differ in any major genetic manner (aside, of course, from mating type), but there are probably many minor genetic differences between them. The association of the enzyme difference with mating type is now undergoing genetic analysis.

It is probable that this difference is a genetical one. The genetic control of melanin production in mammals is well understood.[14] In these cases the changes in pigmentation have been demonstrated to be the result of genetically controlled changes in dopa oxidase activity.[10, 15] A similar situation has been demonstrated in the ascomycete *Glomerella cingulata*, where the tyrosinase concerned seems closely similar to the one demonstrated in the present work.[16] In all of these cases many genes have been demonstrated to influence the enzyme, and it seems that the primary effects of some of these are not directly upon the tyrosinase itself.

In the present instance a situation of a different sort obtains. The mating-type difference is itself a specificity difference of a sort, since the organs of both sexes are present in both mating types. In many respects the mating-type reaction seems similar to the phenomenon of pollen incompatibility, which has in turn been noted to be related to immunological reactions in animals.[1, 17] In the strain we have used, the two mating types also differ in enzyme specificity and antigenic specificity. While the association of these latter differences with the mating-type locus has not yet been investigated, we seem to have a case well suited for a critical test of the hypothesis of the unitary nature of primary gene action.

Summary.—1. The two mating types of Strain 15300 (albino-2), *N. crassa*, differ in ability to produce melanin.

2. This difference has been demonstrated to be due to the presence in 15300*a* of a tyrosinase exhibiting both mono- and polyphenol oxidase activity and its absence in 15300*A*.

3. Both mating types possess suitable substrate for the activity of this enzyme.

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THE NATURE OF LINKAGE VARIATION WITH AGE IN INVERSION HETEROZYGOTES OF DROSOPHILA MELANOGASTER

BY M. WHITTINGHILL AND CLAUDE W. HINTON*

DEPARTMENT OF ZOOLOGY, UNIVERSITY OF NORTH CAROLINA

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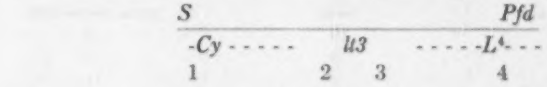
Two kinds of variation in linkage values in Drosophila experiments are well known but poorly understood. One is the change in crossover values with increasing age of the female as shown by Bridges[1]; the other is excessive variation from female to female as pointed out by Gowen.[2] Studies reported in the present paper on an age effect in a special situation may contribute to the interpretation of both problems. They also serve as controls for the irradiation paper which follows.[3]

The chief result of aging is a rapid decrease in recombination values during the first six days of egg-laying, particularly at or near the spindle attachment of the chromosome. After that period smaller changes consist, with variations, of a slight rise and second fall. In recognition of this Bridges[4] has defined as a condition for chromosome mapping the use of

data from young females. From such data inferences are made as to the amount of actual crossing over which has occurred between genes which are assumed to be heterozygous at the time when the germ cells enter meiosis. That this procedure may overestimate the amount of crossing over has been elaborated by the senior author both by reasoning back from observable clustering of the data[5] and by deducing certain consequences of crossing over premeiotically, in gonial cells.[6] The present experiment shows an age effect and clustering attributable to oögonial crossing over in the same body of data, where meiotic crossovers have been greatly reduced or eliminated by inversions.

The age effect in this stock was encountered by accident. In the course of stockmaking by the senior author it was noticed that the infrequent crossovers from *al*[2] *Cy lt*[3] *L*[4] *sp*[2]/*S Pfd* females appeared in early cultures but not in later transfers. Of 14 crossovers in the *Cy-L* interval, 12 were found in the first two cultures and none at all in the last two of a series of five transfers. It was not clear whether this was an accidental result until a larger experiment had been carried out by the junior author and a genuine age effect found.

The experiment was planned with several improvements on the original mass matings. By testcrossing to light males use was made of the *lt* locus, which differentiated the crossovers into two regional kinds. The map order of the loci, the number designations of the crossover regions and the extent of the inversions may be diagrammed as follows:



The spindle attachment lies to the right of *lt*. In the experiment, females were mated individually to these light males, so that the contribution of each family to the totals would be known. Furthermore, the data for each family came separately from five consecutive coded cultures which were not decoded and summed as to families until the experiment was over.

Results and Discussion.—This experiment confirmed the age effect which had been encountered in mass mating. Hence data from both tests have been combined in table 1. In the first two cultures crossovers comprised about 1% of the offspring, in the third they were 0.25% and in the last two transfers practically zero. Almost all of the crossovers came from eggs laid during the first five days of adult life. A chi-square test showed that this was a highly significant departure from the mean of all 11 days. We were therefore dealing with an age effect exhibited by these crossovers.

The data were next examined family by family to see whether the crossovers had appeared at random or in clusters. Random distribution would be in accord with the usual assumption that crossing-over occurs at meiosis,

meaning that no more than one crossover chromatid from each tetrad is recoverable by breeding. Clustering would indicate that there was some gonial influence[7] either (*a*) completed crossing-over in a gonial cell, or (*b*) weakening of a certain place in a chromosome followed later by crossing-over in identical regions in numerous related primary oöcytes. Table 2 shows agglutination of the data, as mathematicians call it, after adding up the cases of crossovers in the two regions of the eighteen families. Although a majority of the 36 cases had no crossovers, paradoxically three-fourths of the crossovers appeared in groups of two or more, as if from the same event of exchange. Furthermore the cases of solitary crossovers, 8, seem too low for a Poisson distribution. Some probabilities will be presented in later paragraphs.

TABLE 1

SUMMARY OF SINGLE CROSSOVERS RECOVERED FROM *Star Pufdi/Curly light Lobe*[4] INVERSION HETEROZYGOTES IN BOTH EXPERIMENTS ACCORDING TO AGE OF FEMALES IN DAYS

Cultures	1	2	3	4	5
Age of female	0–3	3–5	5–7	7–9	9–11
Crossovers	17	20	4	1	1
Total offspring	1747	2062	1677	1752	1886

TABLE 2

AGGLUTINATION IN THE FINDING OF SINGLE CROSSOVER FLIES, FOR EITHER REGION, AMONG 18 FAMILIES OF *S Pfd/Cy lt L*[4] INVERSION HETEROZYGOTES

CROSSOVERS/FAMILY	CASES	TOTAL CROSSOVERS
0	19	0
1	8	8
2	7	14
3	1	3
4	1	4
Totals	36	29

NOTE: Inclusion of seven multiple crossovers would increase the numbers of pairs and especially of triplets.

More detailed familial data appear in table 3. The smallest family, No. 1, contained 4 crossovers in region 2, and these were not all from the same culture, so that they tend to confirm each other. The next smallest family had 3 crossovers in region 3, all found in the same culture. In seven other cases 2 crossovers of a kind were found, and more often than not these were in separate cultures rather than together. Many families, representative in size, failed to have any crossovers in one or both regions. Because of this clustering the crossover values showed great variability among the different families, from about 5 per cent to zero.

One may compute the probability that these are merely chance deviations

from randomness. In region 2 the over-all frequency of crossovers is approximately 0.002, (15/7534 from table 3). In ten families having some 4700 offspring no such crossovers were found, and the probability of this as a random result is 0.998^{4700}, or 0.00008. On the same hypothesis, the probability of drawing a sample of 108 including as many as 4 similar crossovers (Family 1) is 0.0008. Then the joint probability of occurrence of these two aspects of the experiment is 6.4×10^{-8}. Inclusion of the fact that pairs of crossovers were found more often than singles would make it even more improbable that the crossovers in region 2 were independently determined.

TABLE 3

SUMMARY OF TESTCROSS DATA FROM 84 FERTILE, CODED CULTURES AS REASSEMBLED INTO THE ORIGINAL 18 FAMILIES AFTER CLASSIFICATION. FAMILIES LISTED IN ORDER OF INCREASING SIZE

FAMILY NO.	TOTAL PROGENY	SINGLE CROSSOVERS REGION 2	SINGLE CROSSOVERS REGION 3	MULTIPLE CROSSOVERS
1	108	4[a]	1	
2	135	1	3	
3	268	1	0	
4	387	0	1	
5	408	1	2[a]	
6	417	2	0	
7–11	5 × 439.4	0	0	
12	445	2[a]	2	
13	481	0	0	S Cy;[a] lt, wild.
14	490	2[a]	1	
15	520	0	2	Cy L
16	533	2[a]	1	
17	533	0	1	S lt Pfd
18	612	0	0	S lt Pfd L;[a] L.
Totals	7534	15	14	7

[a] Denotes crossovers obtained from two different coded cultures.

Similar calculations for region 3 show that events there are very far from random. The probability that families containing a total of 3975 offspring would have no crossovers, if the latter were randomly distributed at a frequency of 0.183%, is 0.99817^{3975}, which is less than 0.0006. Turning to the other end of the distribution we find that the probability of getting as many as 3 crossovers in a family of only 135 offspring is 0.0157 on the same assumption. The joint probability for the two extremes is more remote, and the further joint probability for region 2 crossovers and region 3 crossovers is 6×10^{-15}.

Up to this point the negative aspects of data of table 3 have been discussed. The kind of non-randomness pointed out may be explained by a

limited amount of gonial crossing over plus gonial multiplication. While this is perhaps the better explanation, there is another interpretation which could reserve crossing over until meiosis. Thus a tendency to produce crossovers near the spindle attachment regardless of region may be possessed by some heterozygotes more than by others. In table 3 one may see that pairs and larger clusters in one region are almost always accompanied by at least one crossover in the adjacent region. This is true in 6 out of 8 families; only females No. 6 and No. 15 had 2 crossovers in one region without any single exchanges in the other. Yet a double crossover, a Curly Lobe fly, appeared in family No. 15 in the same culture with 2 Star Lobe single crossovers. This makes 7 cases of crossing over adjacent to the regions represented by the 8 clusters.

Further evidence for this alternative may be obtained from the multiple crossovers as a whole, which have therefore been included in table 3, even though they are not fully understood. The most plausible multiple crossovers are the Curly Lobe fly just mentioned and the Star light Pufdi of family No. 17. These could be region 2–3 double crossovers, and each was formed in a female which also had one or two single crossovers in region 3. The expectation for these double crossovers in an entire experiment of this size is less than one fly even if figured on the basis of the initial high point of recombination values, 1%, and with no interference. The other multiple crossovers are far less likely than the preceding. The finding of a wild and a light fly, the latter verified by breeding, in the same culture of family No. 13 might be explained as contamination; or these might be triple crossovers, or even quadruples, in some way related to a Star Curly quadruple crossover independently classified in a previous culture. In the subsequent experiment in which similar heterozygotes were irradiated (Hinton and Whittinghill[3]), two more light phenotypes were found in different cultures, so it remains a possibility that this *lt* fly was a 1-2-4 triple crossover. It is indeed strange that there were no single crossover offspring in this family which had two different triples and a quadruple crossover. In one other family, 18, single crossovers were lacking. The only crossovers in this the largest family of all, were a *S lt Pfd* L^4 2–3–4 triple and a verified L^4 1–3 double. Neither kind was detected in the next experiment. However the sporadic nature of the multiple crossovers may be viewed as an extension of the non-randomness already demonstrated among the single crossover types of regions 2 and 3. No single crossovers were recovered from regions 1 or 4 due to the inversions there.

Summary.—There is a decrease in the frequency of crossover offspring from *S Pfd/Cy lt* L^4 females as they age. Crossover values drop practically to zero in seven days, in agreement with the previously known decrease from normal females. Variability in crossover values from family to family was greater than one would expect by chance if each crossover

had been formed separately and independently of every other crossover, the usual assumption about meiosis.[8] Two better explanations have been considered. An entire cluster of crossovers may represent only one actual crossing over in an oögonial cell which was the common ancestor of several eggs. Such an hypothesis is preferred, but an alternate explanation may fit the data of this one experiment without recourse to gonial crossing over. The clustering may begin with a gonial conditioning, such as a permanent weakening of a chromosome, which may influence the location of several later meiotic crossings over. Because identical crossovers and adjacent crossovers tended to be found in the same families in this favorable stock of flies, either of the new hypotheses is appropriate for the present data. Great variability of spontaneous crossover values near spindle attachments would seem to be inescapable in experiments based on the early broods prescribed by Bridges. Hence the collection of data for maps might well be done from a later egg-laying period and, particularly, from large numbers of females to get a measure of the variability.

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* Present address: Biology Division, Calif. Inst. of Technology, Pasadena.

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THE DISTRIBUTION OF X-RAY INDUCED CROSSOVERS FROM CURLY INVERSION HETEROZYGOTES OF DROSOPHILA MELANOGASTER FEMALES

By Claude W. Hinton† and Maurice Whittinghill

Department of Zoology, University of North Carolina

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Introduction.—That crossing over may occur in oögonial cells was suggested by the results of experiments by Whittinghill[1] in which crossing over was induced by x-rays in the *X*-chromosomes of Drosophila females homozygous for the *c3G* asynaptic factor. It had previously been demonstrated that induced crossovers recovered from Drosophila males were of spermatogonial origin.[2, 3] Cooper's observations[4] of chiasmata in gonial cells of both sexes of Drosophila may provide cytological foundation for the hypothesis of gonial origin of some crossovers, particularly those which are x-ray induced. Somatic crossing over, the basis for the occurrence of twin spots in the hypodermis of Drosophila males and females,[5] closely parallels gonial crossing over in the formation of daughter cells which have become homozygous distally. The possible consequences of oögonial crossing over upon linkage data, as recently discussed by Whittinghill,[6] illustrate the need for further investigation of this phenomenon. This paper reports the results of x-ray induced crossing over in Curly inversion heterozygotes of *Drosophila melanogaster* females, and these results are interpreted in relation to normal, or random, meiotic events as opposed to oögonial events of recombination.

Experimental Methods.—Stocks and experimental procedures, with the exception of the treatment employed in this work, were identical with those described in the companion paper[7] in these PROCEEDINGS. Twenty-eight adult virgin females between six and twelve hours old were placed in a 0.7-ml. gelatin capsule and exposed to a total x-ray dose of 2250 r, which was received at the rate of 350 r per minute from a distance of 20 cm. through a 4-mm. aluminum filter from a tube operating at 140 kv. These females were allowed to feed for one day before being testcrossed separately to 4 or 5 *lt*/*lt* males in consecutive half-pint bottles for life. Transfers, without etherization, were made every other day for the first seven cultures and at longer intervals for the final three cultures. As in the former experiment, all cultures were coded so that classifications were performed without bias. Uncertain phenotypes were determined by testcrosses to light homozygotes. Although the spontaneous recombination data of the preceding report have been employed to some extent as controls, accurate comparison of the frequency of crossing over is not possible because of the absence of randomly determined recombination offspring.

The authors have concentrated their study on the actual distribution of induced recombinants in an attempt to detect the place and manner of their origin.

Results.—Although fertility of the irradiated adults was decidedly lower than that of controls, recombination values were greatly increased throughout the egg-laying period (table 1). This persistence of the increase in recombination for as long as 31 days after treatment may have been the result of early as well as late oögonial cells having been affected. Any influence of age on spontaneous recombination is obscured by the much larger effect of the x-rays. This effect included a real peak in recombination between 4 and 8 days after treatment, as shown by the standard errors for cultures 3 and 4 in table 1.

TABLE 1

COMPARATIVE EFFECTS OF AGE (*C*) AND X-RAYS (*X*) ON RECOMBINATION VALUES IN CONSECUTIVE TESTCROSS CULTURES OF *S Pfd/Cy lt L*[4] FEMALES

CULTURE NUMBER	FERTILE FEMALES (*C*)	FERTILE FEMALES (*X*)	TOTAL PROGENY (*C*)	TOTAL PROGENY (*X*)	PER CENT RECOMBINATION (*C*)	PER CENT RECOMBINATION (*X*)
1	18	20	1334	60	0.82 ± 0.24	5.00 ± 2.81
2	18	21	1681	532	0.83 ± 0.22	3.95 ± 0.27
3	18	16	1414	250	0.14 ± 0.10	10.80 ± 1.96
4	18	14	1511	549	0.07	7.47 ± 1.12
5	18	13	1594	379	0.06	5.01 ± 1.12
6		13		631		3.65 ± 0.75
7		12		467		1.93 ± 0.64
8		9		390		2.82 ± 0.84
9		6		427		4.22 ± 0.97
10		1		34		11.76 ± 5.52
Totals			7534	3719	0.38 ± 0.07	4.73 ± 0.35

Three results which are unexpected in random meiotic processes are revealed when the data are recombined into familial groups (table 2). The first notable feature of the table consists of large deviations from the mean total recombination value of 4.73 ± 0.35 per cent. These values range from zero to above 12 per cent. Secondly, the usual assumption of linearly random exchanges is not substantiated due to the wide variations in proportions of region 2 to region 3 crossovers as exhibited chiefly in the families of Females 5, 11 and 13, as compared with 16. Thirdly, pronounced inequalities in complementary crossover classes within a region may be observed in the crossover columns of Females 5, 11, 16 and 23. All of these three forms of variation from female to female have been shown to be expected consequences of oögonial crossing over[1, 6] and may be interdependent to some extent. The most diagnostic of these consequences, and the one which lends itself most readily to analysis, is the extent of imbalance between complementary crossover classes.

TABLE 2

FAMILIAL DISTRIBUTION OF TESTCROSS OFFSPRING FROM IRRADIATED ADULT *S Pfd/Cy lt L*^{s} FEMALES

FEMALE NUMBER	NON-CROSSOVERS		SINGLE CROSSOVERS				TOTAL PROGENY	PER CENT RECOMBINATION
	S Pfd	*Cy lt L*^{s}	REGION 2		REGION 3			
1	96	98	3	0	2	2	201	3.48 ± 1.29
2	128	146	5	2	5	2	288	4.86 ± 1.27
4	86	95	2	3	3	0	189	4.23 ± 1.46
5	274	261	13	7	0	0	556*	3.96* ± 0.83
8	120	135	1	1	1	0	258	1.16 ± 0.67
11	103	85	8	2	0	1	199	5.53 ± 1.62
12	33	25	3	3	1	1	66	12.12 ± 4.03
13	135	136	9	10	4	2	296	8.45 ± 1.62
15	7	8	0	0	1	0	16	6.25 ± 6.06
16	225	233	1	1	12	5	477	3.98 ± 0.90
18	238	230	5	7	6	5	491	4.68 ± 0.95
19	62	59	4	2	3	0	129	6.20 ± 2.12
20	36	49	0	1	1	0	87	2.30 ± 1.67
22	21	33	3	1	1	3	62	12.90 ± 4.26
23	58	62	0	2	5	0	127	5.51 ± 2.02
26	85	91	5	2	2	1	186	5.38 ± 1.65
12 others	39	52	0	0	0	0	91	0
Totals	1746	1798	62	44	46	22	3719	4.73 ± 0.35

* Includes 1 *S lt Pfd* double crossover.

TABLE 3

CHI SQUARE ANALYSIS OF THE DISTRIBUTION OF COMPLEMENTARY CROSSOVERS WITHIN FAMILIES OF IRRADIATED FEMALES

FEMALE NUMBER	REGION 2			REGION 3		
	S lt L^{s}: *Cy Pfd*	*p*	$-\log_e$	*S L*^{s}: *Cy lt Pfd*	*p*	$-\log_e$
1	3:0	0.125	2.080	2:2	0.500	0.693
2	5:2	0.227	1.483	5:2	0.227	1.483
4	2:3	0.500	0.693	3:0	0.125	2.080
5	13:7	0.132	2.025	(0:0)	...	....
8	1:1	0.500	0.693	(1:0)	...	....
11	8:2	0.055	2.904	(0:1)	...	....
12	3:3	0.500	0.693	1:1	0.500	0.693
13	9:10	0.500	0.693	4:2	0.344	1.067
16	1:1	0.500	0.693	12:5	0.072	2.632
18	5:7	0.387	0.950	6:5	0.500	0.693
19	4:2	0.344	1.067	2:0	0.250	1.386
22	3:1	0.313	1.161	1:3	0.313	1.161
23	0:2	0.250	1.386	5:0	0.031	3.478
26	5:2	0.227	1.483	2:1	0.500	0.693
Total $-\log_e$			18.004			16.059
Chi-square	(28 d. f.)		36.008	(22 d. f.)		32.118
Probability			0.154			0.079

Joint Probability = 0.0121

In order to answer the question whether the observed deviations from equality are greater than those allowed by chance, the following analysis was performed. Probabilities of obtaining the observed distribution of complementary crossover classes in each family or of any distribution deviating more in the same direction were computed (table 3). The $-\log_e$ equal to $chi^2/2$ associated with two degrees of freedom, is additive in character,[8] and this allows an over-all probability to be obtained for either region for all families. Since the two regions concerned are on opposite sides of the centromere, one may assume that crossings over occurring simultaneously in these regions are independent of each other. Distributions of 1:0 or 0:1 have been omitted from calculations, for although they add two degrees of freedom to the total, they can contribute no useful information here.

While there are no individual probabilities in the above table which are significant alone, there appears to be a definite excess of distributions of low likelihood as shown by the probabilities of 0.154 and 0.079 for regions 2 and 3, respectively, all families combined. Furthermore, the joint probability for both regions is 0.0121, so that a similar over-all distribution may be expected to occur by chance alone only one time in one hundred like experiments.

Viability differences in crossover chromosomes may sometimes account for numerical differences in complementary crossover classes throughout an experiment; however, this possibility may be reasonably eliminated here. Six additional testcrosses of males whose genotypes corresponded to a combination of complementary crossover chromosomes from region 2 exchanges yielded 706 *S lt* L^4 and 792 *Cy Pfd* flies. This inequality is in the opposite direction from that in the crossover progeny from testcrossed females which gave a 62:44 majority to the *S lt* L^4 class. As there is no reason to suspect that the relative viabilities of the two chromosomes may be reversed depending on whether they originate from sperms or eggs, some cause other than viability differences must be responsible for the observed inequalities in complementary crossover offspring from irradiated females. Similar tests yielding 579 SL^4: 530 *Cy lt Pfd* indicate that larval viability differences played little or no part in the 46:22 result from region 3.

Previous studies of induced crossing over in Drosophila have revealed a tendency of some recombinants to appear simultaneously among the testcross progeny, and several instances of this irregularity occurred in this experiment. A cluster of 6 region 2 recombinants distributed equally was recovered from the third culture of Female 13 among a small brood of 19 flies which included only one crossover from the third region. By comparison, the mean brood size per fertile culture was found to be 33.50 of which 1.58 flies would be expected to be recombinants, assuming a ran-

dom distribution. The fourth culture of Female 4 contained only 3 recombinants, identical region 2 triplets, among a total brood of 42. At this same age, Females 11 and 18 each produced identical quadruplets from region 2, and each set was accompanied by twins of the complementary class; in addition, the latter female had twins from region 3. While these examples are from the two cultures which exhibited the maximum recombination values, clustering was not limited to this period. It is very unusual that although Female 5 produced 7 region 2 recombinants out of 182 offspring in Culture 9 (20–26 days), region 3 recombinants were not represented; the Culture 10 (27–31 days) brood of 34 from this same female included 4 crossovers, all from region 2. Other clusters of lesser magnitude might be cited, and it is possible that some clusters were missed due to splitting them when transferring the cluster-producing female into a new culture.

Two light homozygotes, classified with the broods of Females 18 and 23, have not been included in the data because of the possibility of their being contaminants. Yet, they are not too improbable representatives of quadruple crossing over when compared with similar multiple crossovers which appeared in controls. Furthermore, in order to recover any recombinants from a region included in an inversion, at least double crossing over must occur since single exchanges alone lead to the production of lethal dicentrics and acentric chromosome fragments.[9] One is also reminded that although the Curly inversions do severely limit the frequency of single exchanges in adjacent non-inverted regions, the non-random occurrence of recombinants resulting from such exchanges is not explained by the presence of these inversions.

Discussion.—If an exchange of homologous segments of paired bipartite prophase chromosomes should occur in only a relatively few oögonial cells, and the products of such an exchange be erratically reproduced before the initiation of meiosis, one may well expect the resulting recombination offspring to be distributed non-randomly as recorded in this experiment. Or, as suggested by the distribution of spontaneous recombinants from Curly inversion heterozygotes, oögonial *exchange* of homologous segments may not be necessary for agreement with the results if only a localized weakening of one chromatid at this time may predispose meiotic crossing over at this point after several mitotic divisions have intervened. It seems plausible that these alternatives could be tested by a cytological examination of the first meiotic division, for if exchange occurred prior to this division, the regions distal to the break and including the inversion would frequently become homozygous, allowing normal synapsis in this arm and free chiasma formation.

On either hypothesis, it is necessary to assume unequal multiplication of the affected chromosomes, and the agencies responsible for this may be

several. For example, different rates of division might be conditioned by the exchange products themselves, or could be imposed by chance according to the relative positions the daughter oögonia occupy in the ovary; some of them may be crowded aside from the main line of division either to be lost entirely or relegated to later appearance. However, some causal factor other than larval viability differences must operate preferentially in one direction in order to explain the total inequalities in complementary crossover adults. Cell lethals could presumably explain this if some oögonia become homozygous due to prior exchange.

Applying this idea to the present experiment, we may assume that Star and Curly may become homozygous in adjacent cells after region 2 exchanges, and that Pufdi and Lobe can become homozygous after region 3 exchanges. If any of these mutants are homozygous cell lethals (all are homozygous lethal in larvae), they could not cause more than a 50 per cent reduction of any crossover chromosome, since such chromosomes may segregate into heterozygous situations as frequently as into the homozygous condition. We would then infer from the 46 *S* L^4:22 *Cy* lt^3 *Pfd* ratio of recombinants that Pufdi is completely lethal to cells homozygous for this factor; i.e., in *S Pfd/Cy lt Pfd* gonia. In the case of region 2 exchanges, Curly would appear to be detrimental, although not completely lethal, to oögonial cells homozygous for it as judged from the ratio of 62 *S* lt^3 L^4 to 44 *Cy Pfd* recombinants from this region. While this hypothesis is dependent on oögonial exchange, other possibilities may be found which agree equally well with oögonial weakening and subsequent meiotic crossing over.

Two hypotheses of the mode of action of x-rays in inducing crossing over have appeared in the literature, and these may be briefly considered. In attempting to demonstrate a single mechanism of crossing over for both male and female Drosophila, Friesen[10] found that the Curly inversions did not limit x-ray induced crossing over between black and cinnabar in males as much as in females. On the basis of this evidence, the lack of spontaneous male crossing over was ascribed to an incomplete conjugation of homologous chromosomes (the greater the normal degree of synapsis, the greater the depressing influence of the inversions). Friesen further explained the action of x-rays while increasing recombination to result in "a closer intercourse of the long autosomes (first of all in the central regions)" for both sexes. This hypothesis obviously fails to account for the nonrandom occurrence of recombinants in our experiments, unless it is applied to the chromosomes of gonial cells. Friesen's omission of this application apparently indicates abandonment of his own earlier proposal that crossovers induced in males were of spermatogonial origin.

Another possible mode of action of x-rays in inducing crossing over which more nearly conforms to our experimental results has recently been ad-

vanced by Cooper[4] as a result of observations of chiasma-like configurations in Drosophila gonial cells of both sexes. While it is clear that such chiasmata do not regularly lead to spontaneous recombination, at least in the male, Cooper supposes that these configurations may be competent targets for ionizing particles, and he concludes that x-ray induced crossovers are probably of gonial origin. This would be the place of origin of the crossovers found in this experiment in view of the tendency for complementary crossovers to vary in either direction from the expected.

Summary.—The induced crossover offspring from testcrossed *S Pfd/Cy lt*[1] *L*[4] females x-rayed with 2250 r as adults were so distributed that their meiotic origin is unlikely. The effects of the x-rays were evident for as long as 31 days, with a peak in recombination between 4 and 8 days after treatment. Wide variations from female to female were observed in total per cent recombination, in the relative numbers of recombinants from adjacent regions, and in the distribution of complementary crossover classes within each region. Some tendency toward clustering of the recombinants among the testcross offspring was revealed. These results are indicative of some oögonial influence upon recombinants. This influence may be merely a weakening of oögonial chromosomes followed much later by meiotic crossing over at the weak point, or it may be completion of crossing over in the oögonia. The data on the variable balance of complementary classes favors the latter hypothesis.

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† Present address: Biology Division, Calif. Inst. Tech., Pasadena.

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VARIOUS KERNELS IN THE THEORY OF PARTIAL DIFFERENTIAL EQUATIONS*

BY S. BERGMAN AND M. SCHIFFER

HARVARD UNIVERSITY

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1. We deal with the partial differential equation

$$\Delta u = q(P)u, \qquad \Delta = \frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2} \tag{1}$$

where $q(P)$ is a positive continuously differentiable function of the point $P \equiv (x, y)$ in the closure of a finite domain D of the x, y-plane. We suppose that D is bounded by a set C of analytic curves. We define the bilinear integral

$$E\{u, v\} = \int\int_D [\text{grad } u \cdot \text{grad } v + q\, uv]d\tau, \quad d\tau = \text{area element in } D \tag{2}$$

and observe that $E(u) = E\{u, u\}$ is the energy integral connected with (1).

Let Σ be the linear space of all solutions of (1) with finite energy integral; we may introduce into Σ the metric based on the scalar product (2) and consider Σ as a Hilbert space. Let $N(P, Q)$ and $G(P, Q)$ denote Neumann's and Green's functions of the differential equation (1) with respect to D. One shows easily that for each function $u \in \Sigma$ there holds:

$$E\{N(P, Q), u(P)\} = u(Q), \qquad E\{G(P, Q), u(P)\} = 0. \tag{3}$$

Here and in the following operations, the E-multiplication is always to be understood with respect to the letter common to both factors.

We define the two kernels

$$K(P, Q) = N(P, Q) - H(P, Q), \qquad L(P, Q) = N(P, Q) + G(P, Q). \tag{4}$$

$K(P, Q)$ is as function of each variable P an element of Σ; in view of (3) it satisfies the identity

$$E\{K(P, Q), u(P)\} = u(Q), \qquad u \in \Sigma, \tag{3'}$$

i.e., it is the reproducing kernel of the class Σ. By Schwarz' inequality

$$u(Q)^2 \leq K(Q, Q) \cdot E(u), \tag{5}$$

i.e., the kernel K provides at each point $Q \in D$ an estimate for the value of u in terms of its norm in Hilbert space. The inequality is the best possible, equality holds for $u(P) = K(P, Q)$. If a set of functions $\{u_\nu(P)\}$ forms a

complete orthonormal set in Σ, we can develop $K(P, Q)$ into the Fourier series

$$K(P, Q) = \sum_{\nu=1}^{\infty} u_\nu(P)u_\nu(Q). \tag{6}$$

This property provides a useful numerical approach to the theory of Green's and Neumann's functions [1, $a - d$].

The kernel $L(P, Q)$ has also the reproducing property under scalar multiplication. It does, however, not belong to Σ because of its singularity for $P = Q$ and has, therefore, not the same importance in the theory of orthogonal solutions of (1). It was originally introduced for reasons of formal symmetry. See reference 3. Its real significance will be shown in the sequel.

2. Let $D_1 \supset D$ and suppose that $q(P)$ is positive and continuously differentiable in D_1. Denote by N_1, G_1, K_1, L_1 the corresponding Neumann's and Green's functions and their combinations with respect to the domain D_1. Let for $P, Q \in D$

$$k(P, Q) = K(P, Q) - K_1(P, Q), \qquad l(P, Q) = L(P, Q) - L_1(P, Q); \tag{7}$$

denote finally by D_0 the difference region between D_1 and D and let $E_0\{u, v\}$ be the integration defined in (2) but extended over D_0. With these notations, we state the following result which is an easy consequence of the boundary behavior of Green's and Neumann's functions:

$$E\{l(P, Q), l(P, R)\} = k(Q, R) - E_0\{L_1(P, Q), L_1(P, R)\}. \tag{8}$$

Let P_i be an arbitrary set of N points in D and x_i be N arbitrary real numbers. We derive from (8) the identity

$$\sum_{i,k=1}^{N} K(P_i, P_k)x_ix_k - \sum_{i,k=1}^{N} K_1(P_i, P_k)x_ix_k = E_0\{\sum_{i=1}^{N} L_1(P, P_i)x_i\} + E\{\sum_{i=1}^{N} l(P, P_i)x_i\}. \tag{8'}$$

Since the right-hand side of (8') is non-negative we conclude that the quadratic forms based on the K-kernels decrease monotonically with increasing domain.

Suppose that for the domain D_1 the fundamental functions N_1 and G_1 are known. In this case, (8') leads to an estimate for the unknown kernels of all subdomains $D \subset D_1$:

$$\sum_{i,k=1}^{N} K(P_i, P_k)x_ix_k \geqslant \sum_{i,k=1}^{N} K_1(P_i, P_k)x_ix_k + E_0\{\sum_{i=1}^{N} L_1(P, P_i)x_i\} \tag{8''}$$

where all right-hand terms are known.

3. The kernel $K(P, Q)$ becomes logarithmically infinite if both argument points tend to the same point P_0 on the boundary C of D. The same holds then for the kernel $k(P, Q)$ since $K_1(P, Q)$ is continuous in the interior of D_1. It can be shown that $l(P, Q)$ is continuously differentiable in the closed region $D + C$. It thus appears that among the difference kernels k and l, the l-kernel is the more regular while among the K and L-kernels the K-kernel has the better regularity character. By means of identity (8), we are now able to investigate easily the singularity of $k(P, Q)$ on the boundary C of D. In fact, $k(Q, R)$ and $E_0\{L_1(P, Q), L_1(P, R)\}$ have there the same asymptotic behavior, since their difference is regular in the closed region.

The linear transformation of the Hilbert space Σ into itself

$$T_0(P) = E\{l(P, Q), u(Q)\} \tag{9}$$

can be shown to be completely continuous. Thus, the eigenvalues of the integro-differential equation

$$u_\rho^*(P) = \lambda_\rho E\{l(P, Q), u_\rho(Q)\} \tag{10}$$

form a discrete spectrum. If we also permit the eigenvalue $\lambda = \infty$, we can select a system of eigenfunctions $\{u_\rho(P)\}$ of (10) which form a complete orthonormal set in Σ. In terms of this set, we have the Fourier developments

$$K(P, Q) = \sum_{\rho=1}^{\infty} u_\rho(P)u_\rho(Q), \qquad l(P, Q) = \sum_{\rho=1}^{\infty} \frac{1}{\lambda_\rho} u_\rho(P)u_\rho(Q), \tag{11}$$

converging uniformly in each closed subdomain of D. It can be shown that all eigenvalues λ_ρ satisfy the inequality

$$|\lambda_\rho| > 1. \tag{10'}$$

4. We assume again that the fundamental functions of D_1 are known. Then the expression

$$K_1(Q, R) + E_0\{L_1(P, Q), L_1(P, R)\} = \Gamma(Q, R) \tag{12}$$

is to be considered as an elementary quantity, obtainable from N_1 and G_1 by the processes of algebra, differentiation and integration. We can reduce the solution of the boundary value problems with respect to (1) and the domain $D \subset D_1$ to sequences of such elementary operations.

We remark from (8) and (11) that

$$M(Q, R) = K(Q, R) - \Gamma(Q, R) = \sum_{\rho=1}^{\infty} \frac{1}{\lambda_\rho^2} u_\rho(Q)u_\rho(R). \tag{13}$$

This shows that the positive-definite kernel $M(Q, R)$ has its lowest eigenvalue larger than 1. Let us consider the integral equation for $K(Q, R)$ with kernel $M(P, R)$.

$$K(Q, R) - E\{M(P, R), K(P, Q)\} = \Gamma(Q, R) \tag{14}$$

which is an immediate consequence of the reproducing property of the K-kernel. Since the lowest eigenvalue of M is larger than 1 we can solve (14) by means of the Neumann series development:

$$K(Q, R) = \sum_{\nu=0}^{\infty} E\{M^{(\nu)}(P, Q), \Gamma(P, R)\} \tag{15}$$

where the iterated kernels $M^{(\nu)}(P, Q)$ are defined by

$$M^{(\nu)}(P, Q) = E\{M^{(\nu-1)}(T, P), M(T, Q)\}, \qquad M^{(0)}(P, Q) = K(P, Q). \tag{15'}$$

Introducing similarly the νth iterated kernel $\Gamma^{(\nu)}(Q, R)$ based on $\Gamma^{(1)} = \Gamma$ as defined by (12), we may transform (15') by means of (13) into

$$K(Q, R) = \sum_{\nu=0}^{\infty}\left[\sum_{\rho=0}^{\nu}\binom{\nu}{\rho}(-1)^{\rho}\Gamma^{(\rho+1)}(Q, R)\right]. \tag{16}$$

Thus, $K(Q, R)$ is developed into an infinite series of terms each of which can be obtained by elementary processes. Let $K_n(Q, R)$ be the kernel obtained by summing in (16) only for $0 \leq \nu \leq n$; the difference kernel $K - K_n$ can be shown to be positive-definite so that each approximate calculation of K leads to an inequality for the K-kernel. (8^{ν}) is the inequality obtained by taking $n = 0$.

5. Let us suppose that the curve system C is obtained from the analytic boundary curves C_1 of D_1 as follows: If s is the length parameter of C_1, we define an analytic non-negative function $\varphi(s)$ on C_1; we erect at each point $P_1 \in C_1$ the interior normal and proceed on it by an amount $\epsilon\varphi(s) = \delta\nu$; where $\epsilon \leq 0$ is a smallness parameter. The end-points $P(s)$ thus obtained form, for ϵ small enough, a permissible C system. We can show that for all eigenvalues of (10)

$$\left|\frac{1}{\lambda_{\rho}}\right| = O(\epsilon) \tag{17}$$

holds. This implies by (13)

$$M(Q, R) = O(\epsilon^2), \qquad M^{(\nu)}(Q, R) = O(\epsilon^{2\nu}), \tag{18}$$

uniformly in each closed subdomain of D.

Using the estimate (18) in (16), we find after elementary transformations

$$K(Q, R) - K_1(Q, R) = \int_C [\text{grad } K_1(P, Q) \cdot \text{grad } K_1(P, R) + q(P)K_1(P, Q)K_1(P, Q)]\delta\nu_P ds_P + O(\epsilon^3) \quad (19)$$

where the estimate $O(\epsilon^3)$ holds uniformly in each closed subdomain of D. One also easily derives from (16) and (18)

$$L(Q, R) - L_1(Q, R) = \int_C [\text{grad } K_1(P, Q) \cdot \text{grad } L_1(P, R) + q(P)K_1(P, Q)L_1(P, R)]\delta\nu ds + O(\epsilon^3). \quad (20)$$

These formulas are equivalent to the variational formulas for Green's and Neumann's functions which were given first by Hadamard in the case of Laplace's equation [2]:

$$\delta G(Q, R) = -\int_C \frac{\partial G(P, Q)}{\partial \nu_P} \frac{\partial G(P, R)}{\partial \nu_P} \delta\nu_P ds_P \quad (21)$$

$$\delta N(Q, R) = \int_C [\text{grad}_P N(P, Q) \cdot \text{grad}_P N(P, R) + q(P)N(P, Q)N(P, R)]\delta\nu_P ds_P. \quad (21')$$

The significance of the series development (16) now becomes evident; it is a development of the functional $K(Q, R)$ of the domain D in powers of the Fréchet distance from the known domain D_1. The variational formulas (19–21′) stand to this series in the same relation as do the expressions for differentials of functions of finitely many variables to the Taylor series expansions.

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(*c*) "Kernel Functions in the Theory of Partial Differential Equations of Elliptic Type," *Duke Math. J.*, **15**, 535–566 (1948).

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MULTIPLICATIVE TRANSFORMATIONS

By D. G. Bourgin

Department of Mathematics, University of Illinois

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Distributive operations over a linear space have been studied intensively in recent years. There has been comparatively little attention paid to the closely correlated considerations attached to multiplicative transformations. We shall be concerned throughout with the normed rings of real-valued continuous functions over compact sets. Perhaps the most interesting of our results for application in other fields are in connection with our definition of *vital* sets and Theorems 2 and 3. This last theorem seems unexpected for with no denumerability restrictions on neighborhood bases it asserts the maps of elements of our normed ring by a multiplicative functional are completely determined by the element values on a fixed closed countable set. Other topics cover deviations from strict multiplicativity and their consequences.

We use *countable* to cover either finite or denumerable. The complement of a set Q is written $\tilde{Q}$. $C(S)$ is the usual normed ring of the real-valued continuous functions over compact S. We write $C_0(S)$ for the multiplicative semigroup of non-negative elements of $C(S)$ and $C_+(S)$ for the multiplicative group of positive elements. We designate the ring of real numbers by R whence the notations R_0 and R_+ are clear. The elements of $C(S)$ are denoted by small letters. Under the single-valued transformation T on $C(S_1)$ to $C(S_2)$ we shall use the capitalization of the small letter to indicate the correspondent. Thus $Tx = X$. We employ s and t for the points of S_1 and of S_2. Write $\Delta(xy)$ for $\|Txy - Tx\,Ty\|$ and $|x|_t$ for $\min_{S_t} |x(s)|$. If $|x|_t > 0$, x is regular. We indicate various types of non-multiplicativity [cf. reference 1] by saying T satisfies (A) or (B) or (C) where (A): $\Delta(xy) \leq \delta|x|_t\,|y|_t$, $\delta > 0$ (B) $\Delta(xy) = 0$ for $|xy|_t = 0$ and (C) $\Delta(xy) \leq \delta|xy|_t + \epsilon$.

The letters G and F with or without scripts invariably designate open and closed sets, respectively. If $\phi \neq F \subset G$ then since our compact S must be normal, functions $x(s)$ in $C(S)$ exist with $x|F = a$, $x|\tilde{G} = b > a \geq 0$ and $a \leq x(s) \leq b$. We use the notation $U(F, G;\ a, b)$ for one of these functions chosen arbitrarily. For $(a, b) = (a, 1)$ and $(0, 1)$, respectively, we write $U(F, G;\ a)$ and $U(F, G)$.

THEOREM 1. *If T satisfies (A) and S_1 has at least two points then T is actually multiplicative.*

If the assertion were untrue then for some x and y specified by primes, $(T(x'y') - Tx'\,Ty')(t) = D(t)$ does not vanish for $t \in Q$. Let $x_N(s) = U(F, G;\ N^{-1}, N)$ and let $y_N(s)$ be the reciprocal of $x_N(s)$. Observe

$$\|Tx_N(Tx'\,Ty' - Tx'y')\| \leq \|\,Ty'(Tx_NTx' - Tx_Nx')\,\| + \Delta((x_Nx')y') +$$
$$\Delta((x'y')x_N) \leq \frac{\delta}{N}\,[|x'|_t\|Ty'\| + |x'y'|_t + |y'|_t|x'|_t]. \quad (1.0)$$

Therefore, wherever $D(t) \neq 0$,

$$0 \leq |(Tx_N)(t)| \leq \alpha/N|D(t)|, \qquad \alpha \geq 0. \quad (1.01)$$

We have need of the following inequalities, valid when $t \in Q$.

$$\|(Tx'y' - Tx_Nx')Ty_Ny'\| \leq a/N^2,$$
$$\|(Tx'x_N - Tx'Tx_N)Ty'Ty_N\| \leq \|Ty'(Tx'x_NTy_N - Tx')\| \leq b/N^2,$$
$$|(Tx'x_N(Ty_Ny' - Ty'Ty_N))(t)| \leq |(Tx'x_N)(t)|\,|y'|_t\,\delta/N \leq c/N^2,$$

where the last is established by replacing x_N in (1) and (1.01) by (x_Nx'). Thus $|D(t)| \leq (a + b + c)/N^2$ in Q. That is to say $D(t) = 0$ everywhere, in contradiction with the assumed non-multiplicativity of T for the choice x', y'. An example[1] shows the theorem may be invalid if S_1 has just one point.

Let $\mathfrak{F}$ be a field of sets containing all closed sets (though similar concepts have merit with a less specialized $\mathfrak{F}$). Let P and P' designate mutually inconsistent set properties. We require: (1) If the sets A and B are in $\mathfrak{F}$ and $A \subset B$ then B has P' implies A has P'. (2) If A and B have P' then $A \cup B$ has P'. The set A is *vital* (with respect to P, P' and $\mathfrak{F}$) if $A \in \mathfrak{F}$ and if for every partition of A into disjunct sets B_1 and B_2 of $\mathfrak{F}$, B_i has P and $B_{i+1 \bmod 2}$ has P'.

THEOREM 2. *If (a) the null set and single points have P', (b) every set which has P has a compact subset which has P and (c) S is compact, then S contains no vital sets.*

Suppose to the contrary A is vital. If C_1 and C_2 are subsets of A in $\mathfrak{F}$ which have P then $C_1 \cap C_2$ has P. Otherwise $C_1 \cup (C_1 \cap C_2)$ would have P' by (2) and so C_2 would have P' by (1). By induction, if the sets C_i of $\mathfrak{F}$ have P then $\bigcap_{i=1}^{i=N} C_i$ has P whence according to (a) the closed sets in A which have P have F. I. P. (finite intersection property). Let $\{Q_\alpha\}$ be the totality of subsets of A in $\mathfrak{F}$ which have P and let $\{F_\beta\}$ be the subcollection of closed sets in $\{Q_\alpha\}$. Then by virtue of (b) and (c) and the F. I. P. for $\{F_\beta\}$, $\bigcap F_\beta \supset \bigcap Q_\alpha \supset \bigcap F_\beta \Longrightarrow \bigcap Q_\alpha = \bigcap F_\beta = R \neq \phi$. Suppose $x_0 \in R$. Then the dissection x_0, $A - x_0$ implies by (a) that $A - x_0$ has P whence $A - x_0 \in \{Q_\alpha\}$ and so $x_0 \notin R$ which is absurd. Theorem 2 is still valid for a locally compact S.

We shall call a completely additive, real-valued set function $\mu(Q)$ defined on the Borel sets of S, a B measure and we shall say μ is *continuous* if $\mu(Q) = 0$ for all countable sets Q. Suppose P stands for $\mu(A) = \delta > 0$ and P' for $\mu(A) = 0$. Take $\mathfrak{F}$ as the Borel field. Then A is vital if for

all dissections of A into disjunct Borel sets B_1 and B_2, $\mu(B_i) = 0$, $\mu(B_{i+1 \bmod 2}) = \mu(A) = \delta$. (The term "atom" has been used for a somewhat less restricted situation.)

$M(x)$ is a continuous multiplicative functional on $C(S)$ to R if $M(xy) = Mx\,My$ and $\|x^n - x\| \to 0$ implies $M(x^n) \to Mx$. We tacitly *bar* $M(x) \equiv 0$ *or* $M(x) \equiv 1$ *in the sequel.*

THEOREM 3. *If $M(x)$ is a continuous multiplicative functional on $C(S)$ to R, S compact, then there exists a countable closed set D in S independent of x such that $M(x)$ is completely determined by the values of $x(s)$ on D.*

We remark first that on the one hand $M|x|$ is non-negative since $|x|$ is a square and on the other $(M|x|)^2 = M(x^2) = (Mx)^2$ whence $|M(x)| = M|x|$. Hence we can consider Mx for x restricted to $C_0(S)$ in place of $|M(x)|$. Now M is a continuous multiplicative functional on $C_0(S)$ to R_0 and *a fortiori* on $C_+(S)$ to R_+. We sometimes indicate the domain of M by writing M_0 and M_+, respectively. We may consider M_0 as an extension of M_+. This simple observation actually points the way to the proof of our theorem.

If $x \in C_+(S)$ then $x(s) = \exp x'(s)$, where $x'(s) = \log x(s)$. We define the additive functional F by

$$F(x') = \log M(e^{x'}). \tag{3.0}$$

In $C_+(S_1)$, $\|x'^n - x'\| \to 0$ implies $\|x'^n - x'\| \to 0$.

Let e be the function identically 1 on S. Then $M(x) = M(e)\,M(x)$ for all x whence $M(e) = 1$. Therefore $M(x^{-1}) = (M(x))^{-1}$ for regular x. For any integer k and $x \in C(S)$, we have $M(x^k) = (M(x))^k$ whence using $y = x^k$, $(M(y)^{1/k}) = (M(y))^{1/k}$. Accordingly $(M(y)^{l/k}) = (M(y))^{l/k}$. In short $(M(x)^r) = (M(x))^r$ for r rational but not necessarily positive. If $r \to \gamma$, then evidently $\|x^r - x^\gamma\| \to 0$ and so, for any real number γ,

$$M_+(x^\gamma) = (M_+(x)^\gamma). \tag{3.01}$$

These facts show that F is a linear functional on $C(S)$. Therefore $F(x')$ has the representation,[2]

$$F(x') = \int_S x'(s)\,d\mu,$$

where μ is a *regular* B measure with $\mu(S) < \infty$, uniquely determined by F.

$$M_+(x) = \exp \int_S \log x(s)\,d\mu. \tag{3.02}$$

The representation (3.02) is applicable to M_0 if we observe that M_0 is continuous so $M_+(x + \epsilon_n) \to M_0(x)$ for $\epsilon_n \downarrow 0$. Thus if $\int_S \log x(s)\,d\mu$ becomes negatively infinite it is merely necessary to set $M_0(x) = 0$.

Our key assertion is that the B measure μ is concentrated on a closed countable point set D. We assume the assertion false and derive a contradiction.

First we remark that the measure $\mu = \mu_1 - \mu_2$, where μ_1 and μ_2 are non-negative regular B measures. The continuity restriction on $M_0(x)$ requires that $M_0(x)$ be bounded. If $\mu_2 \not\equiv 0$ then for some set A, $\mu(A) \leq -\delta < 0$. We have then by the regularity of the measure that a closed set F and an open set G exist with $F \subset A \subset G$ and $|\mu(B_1) - \mu(B_2)| < \delta/2$ for any two Borel sets boxed between F and G. Let $x_0 = U(F, G; \epsilon)$. Let $\{\lambda_n\}$ be a monotone increasing positive sequence with limit 1. Let $x_n = |x_0 - \lambda_n \epsilon|/1 - \lambda_n\epsilon$. Then $\int \log x_n \, d\mu \geq -(\log 2\epsilon + \log (1 - \lambda_n))\delta/2$ for $n \geq N$, or $M(x_n) \geq (2\epsilon(1 - \lambda_n))^{-\delta/2}$. Plainly the sequence x_n converges but $M(x_n)$ does not. Accordingly $\mu_2 \equiv 0$ and the B measure μ is actually *non-negative*.

Next let $D = \{s_i\}$ be the set of points such that $\mu(s_i) > 0$. Evidently D is countable. Let ν be the completely additive, non-negative regular set function on the Borel sets of S, defined by

$$\nu(A) = \mu(A) - \sum \mu(s_i | s_i \in D \cap A). \tag{3.03}$$

Then there are no ν vital sets for in Theorem 2 (a) is obviously satisfied since $\nu(A)$ is continuous and the regularity of ν shows (b) is also. This sharpens the Kakutani representation[3] for linear functionals in $C(S)$. We digress to remark that for a *locally compact space* Theorem 2 implies the non existence of a two valved regular completely additive Borel measure vanishing on points if the measure and regularity are based in the usual way on compact subsets. (If however F and the notion of regularity are based on the closed sets such a measure can exist and Oxtoby has communicated an example to the writer).

Consider a sequence of positive numbers $\{a_n\}$ such that $\sum a_n < \infty$ and write Π_i^n for $(\Pi_{j=i}^{j=n}(1 + a_j))^{-1}$ subject to $\Pi_i^n = 0$ for $i < 0$, $\Pi_i^n = 1$ for $i > n$ and $\Pi_i = \Pi_i^\infty$. Define $(1 + i)^2 d_i$ as $1 - (\Pi_{i+1})$.

Admit $\nu(S)$ is positive. Suppose $\mu(G \cap D) = a$, $\nu(G) = b > 0$. Take an arbitrary positive integer m. Write $D^n = \{s_i | i \geq n\}$, $E^n = D - D^n$. For $n \geq N$ plainly $\mu(D^n) < a/2m$. Let $G_0 = G - E^n \cap G$. Since ν is continuous and admits no ν vital sets, G_0 can be partitioned into $m + 1$ disjunct Borel sets of positive ν measure whence one, say B, satisfies $0 < \nu(B) \leq b/n + 1$. In view of the regularity of ν some G_∞ and G' exist with $B \subset G_\infty \subset G_0$ and $G' \subset \bar{G}' \subset G_\infty$ with $0 < \nu(G') < \nu(G_\infty) < b/m$. Hence $\mu(G') \leq \nu(G') + \mu(G_0 \cap D) \leq (a + b)/m = \mu(G)/m$. Accordingly we can determine a sequence

$$G_0 \supset \bar{G}_1 \supset G_1 \supset \ldots \supset \bar{G}_i \supset G_i \supset \ldots \tag{3.04}$$

with

$$\mu(\bar{G}_k) < d_k \mu(G_{k-1}). \tag{3.05}$$

Consider the sequence defined by induction $x^1(s) = U(\bar{G}_1, G_0), \ldots$ $x^{n+1}(s) = (a_n + x^n(s))(1 + a_n)^{-1} U(\bar{G}_{n+1}, G_n), \ldots$. For $s \in \bar{G}_{i-1} \cap \bar{G}_i$

$$1 - \Pi_i^n \leq x^{n+1}(s) \leq 1 - \Pi_{i-1}^n. \tag{3.06}$$

If $n > m$, $x^{n+1}(s) - x^{m+1}(s) \leq (1 - \Pi_m)$. Accordingly x^n converges uniformly to a function $z \in C_0(S)$. Since for fixed s $\{x^n(s)\}$ is monotone nondecreasing the zeros of $z(s)$ are contained in $\bigcap_{i=1}G_i$ and therefore in $\bigcap_{i=1}\bar{G}_i$. Clearly $\mu(\bigcap \bar{G}_i) \leq \mu(\bar{G}_n)$ for all n and so $\mu(\bigcap \bar{G}_i) = 0$. From (3.06) and $|\log a| < a^{-1}$ when $0 < a$, $|\int \log z(s)\, d\mu| < \sum(1 - \Pi_i)^{-1} \mu(\bar{G}_{i-1}) \leq \sum_{i=1} i^{-2}$. In short $M(z) \neq 0$. However, since x^n vanishes on a set of positive measure $0 = Mx^n \to M(z)$. The contradiction establishes that $\nu(S) = 0$.

We show D is closed. Suppose s_0 is a cluster point of D with $s_0 \bar{\in} D$, $\mu(s_0) > 0$. This implies the existence of x in $C_0(S)$ with $x(s_0) = 0$ and $M(x) > 0$. To show this observe that since $\{\mu(s_i) | s_i \in D\} \in l_1$ it is easy to demonstrate that for some strictly monotone increasing unbounded positive sequence $\{\lambda_n\}$, $\{\lambda_i \mu(s_i)\} \in l_1$. Let $y(s) = \sum U(s_0, s_n)/2^n$. Then $y \in C_0(S)$, $y(s_0) = 0$ and $2^{-i} \leq y(s_i) \leq 1$. Let h define a 1-1 order preserving correspondence between the denumerable closed subsets $0 \cup \{2^{-n} | n = 0, 1, \ldots\}$ and $0 \cup \{\exp - \lambda_n\}$ of the unit interval with $h(0) = 0$, $h(2^{-n}) = \exp - \lambda_n$. Then plainly h extends to an order preserving homeomorphism H of the unit interval into itself. Define $x(s)$ as $Hy(s)$. Note $x(s_0) = 0$ and $\exp - \lambda_i \leq x(s_i) \leq 1$. Accordingly $M(x) \geq \exp - \sum \mu(s_i)\lambda_i > 0$. Let $V_n = \{s | x(s) < \epsilon_n\}$, $W_n = \{s | x(s) \leq \epsilon_n/2\}$, where $0 < \epsilon_{n+1} < \epsilon_n/2$. Let $x^n(s) = U(W_n, V_n)x(s)$. Plainly $D \cap W_n \neq \phi$ and therefore $Mx^n = 0$. We next note $|x^n(s) - x(s)| < \epsilon_n$. Hence $x^n \to x$ and so $Mx = 0$. This contradiction establishes that actually $s_0 \in D$.

The representation of $|Mx|$ is then plainly

$$|M(x)| = \Pi |x(s_i)|^{\mu(s_i)}. \tag{3.07}$$

Since $\int \log x(s)\, d\mu$ is of course absolutely convergent the infinite product is absolutely convergent. Incidentally note D is not vacuous.

We finish our proof by showing sign $M(x)$ is determined by the values of x on D also. We need only disprove the possibility that $x_1(s) = x_2(s)$ on D but sign $M(x_1) = 1$, sign $Mx_2 = -1$. If $z_\lambda = \lambda x_1 + (1 - \lambda)x_2$, $0 \leq \lambda \leq 1$, $M(z_\lambda)$ is continuous in λ and since $|M(z_\lambda)| = |M(x_i)|$, $i = 1, 2$, sign Mz_λ must vary continuously from -1 to 1 which is absurd since the sign ( ) function can assume only 3 values.

Accordingly, taking into account also the lemma below, sign $M(x)$ is 0 for non-regular x or is given by any continuous homomorphism, h (sign $x(D)$), of the multiplicative group of the obvious equivalence classes (according to sign) of the regular elements of $C(D)$, D a denumerable

compactum, to the multiplicative group with two elements $(1, -1)$. A natural designation for D is therefore that of "determining set." We remark that if the range of M is K the complex number field then the determining set D is again a denumerable compactum. Indeed (3.07) retains its validity for $M|x|$ provided $\mu(s|D)$ is replaced by $\mu_1(s|D) + i\mu_2(s|D)$ where $\mu_1(s_i)$ is positive but $\mu_2(s_i)$ may be of either sign or 0.

LEMMA. *A denumerable closed subset D of a compact (Hausdorff) set S is a compactum in the induced topology.*

We remark that in view of well-known theorems it is sufficient to demonstrate a countable neighborhood basis in D at each point s of D. If $\bar{s}$ is isolated, then, for some neighborhood U in S, $U\bar{s} \cap D - \bar{s} = \phi$ and $\bar{s}$ is itself a neighborhood. If s is not isolated then for each s distinct from $\bar{s}$ there is a neighborhood of $\bar{s}$ in S, which does not contain s. Denote the totality of these neighborhoods by $\{U^i | i = 1, 2, \ldots\}$. Choose the neighborhoods V^i so that $\bar{s} \subset V^i \subset \bar{v}^i \subset U^i$ and let $W^i = \bigcap_{j=1}^{j=i} V^j$. The corresponding neighborhoods in D are denoted by the corresponding small letters. Thus $w^i = w^i \cap D$, $v^i = V^i \cap D$, etc. We assert $\{w_i\}$ is a neighborhood basis at $\bar{s}$. Otherwise for some neighborhood of $\bar{s}$, say g, $w^i \cap \bar{g} \neq 0$ for all i. Select $s^i \in w^i \cap \bar{g}$. Compactness guarantees a cluster point $z \in D$. However $z \in g$ since s^i is foreign to g. Thus $z \neq \bar{s}$ and since $\bar{s} = \bigcap u^i \supset \bigcap \bar{v}^i$ we infer $z \in \bar{v}^i$ for some i. Therefore some neighborhood of z includes no s^j for $j \geq i$ in contradiction with the fact that z is a cluster point.

If $x \in C(S)$ then $x|D \in C(D)$. Tietze's theorem shows every continuous function on D is extensible to a continuous function on S. It follows that the representation problem for sign $M(x)$ is reducible to the case of continuous functions on a countable compactum.

THEOREM 4. *If S_i is compact $i = 1, 2$, and if (a) T satisfies B and (b) at least one non-regular element of $C(S_1)$ is not mapped into the zero element of $C(S_2)$ then, if (c) all of $C(S_2)$ is covered, T is multiplicative.*

Let $Q = \{t | (Tx)(t) \neq 0$ for some non-regular $x \in C(S_1)\}$. It is known [Lemma 7, reference 1] that $(Tx)(t)$ is multiplicative for $t \in Q$. (This establishes the theorem if S_2 has just one point.)

We contend that for $t_1 \in Q$, $(Tx)(t_1) = 0$ implies x is non-regular. Otherwise x^{-1} exists and for any $z \in C(S_1)$, $(Tz)(t_1) = (Txx^{-1}z)(t_1) = (TxTx^{-1}z)(t_1) = 0$. This denies (c).

Suppose T is not multiplicative then $\tilde{Q}$ contains a point designated by t_0. Let $t_1 \in Q$. Consider $Y \in C(S_2)$ with $Y(t_1) = 0$ and $Y(t_0) \neq 0$. Suppose $Y = Ty$. Obviously y is regular since $t_0 \in \tilde{Q}$. On the other hand $(Ty)(t_1) = 0$ requires y be non-regular. The contradiction establishes the theorem. Examples[1] show that neither (b) nor (c) can be dispensed with. Observe (a), (b), (c) are not consistent if S has just one point.

We now consider relation (C). (The parenthetical remark on page 392

of reference 1 referring to the connection between the relation referred to in that paper by A and our present C is not quite correct without further restriction.)

LEMMA. *If T satisfies (C) and for every $t_1 \in S_2$ there exists a sequence of functions w_N in $C(S_1)$ possibly dependent on the choice of t_1 such that $(Tw_N)(t_1) \to \infty$ and if $|xy|_t = 0$ then T is multiplicative on xy.*

Suppose $|y|_t = 0$ then

$$|((Txy - Tx\,Ty)Tw_N)(t_1)| \leq \Delta((xy)w_N) + \Delta(x(yw_N)) + \|Tx(Tw_N - TyTw_N)\| \leq \epsilon\,(2 + \|Tx\|).$$

In short $|(Txy - Tx\,Ty)(t_1)| \leq \epsilon\,(2 + \|Tx\|)/|(Tw_N)(t_1)| \to 0$.

THEOREM 5. *If T satisfies C and is $1 - 1$ on $C(S_1)$ onto $C(S_2)$ then S_1 and S_2 are homeomorphic.*

Indeed since the mapping is "onto" the sequence w_N exists for every choice of t_1 in S_2 and *hence* $|xy|_t = 0$ *implies* $Txy = Tx\,Ty$. Theorem 4 now guarantees T is actually multiplicative and our result follows from Theorem 6 of reference 1 or from reference 3.

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*EXTENSIVE GAMES**

BY H. W. KUHN

PRINCETON UNIVERSITY

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In the mathematical theory of games of strategy as described by von Neumann and Morgenstern,[1] the development proceeds in two main steps: (1) the presentation of an all-inclusive formal characterization of a general n-person game, (2) the introduction of the concept of a pure strategy which makes possible a radical simplification of this scheme, replacing an arbitrary game by a suitable prototype game. These two forms have been given technical names by von Neumann and Morgenstern, who called them the *extensive* and the *normalized* forms of a game. As they have noted, the normalized form is better suited to the derivation of

general theorems (e.g., the main theorem of the zero-sum two-person game), while the extensive form exposes the characteristic differences between games and the decisive structural features which determine those differences. Since all games are found in extensive form, while it is practical to normalize but a few, it seems reasonable to attack the completion of a general theory of games in extensive form. This note presents two new results in this theory which appear to have far-reaching consequences in the computational problems of normalized games. These results are cast in terms of a new formulation of the extensive form which seems to have intuitive advantages over that used by von Neumann.[2]

In order to engage in a precise discussion, it will be necessary to clarify certain concepts associated with a game which are confused and ambiguous in common use. We will use these terms in essentially the same manner as von Neumann and Morgenstern. A *game* is simply the set of rules which define it, while every particular instance in which a game is played from beginning to end is called a *play* of that game. A similar distinction is drawn between the occasion of the selection of one among several alternatives, to be made by one of the players or by some chance device, which is called a *move* and the actual selection in a particular play which is called a *choice*. Thus, a game consists of a set of moves in some order (not necessarily linear!), while a play consists of a sequence of choices.

1. The Extensive Form.—

Definition: A general n-person game Γ is a finite tree K imbedded in an oriented plane with the following specifications:

(1) A distinguished vertex O.

(*Terminology:* The *alternatives* at a vertex P are the edges e incident to P and lying in components not containing O if we cut K at P. If there are j alternatives at P, then we index these by the integers $1, \ldots, j$, circling P in the positive sense. At the vertex O, the first alternative may be assigned arbitrarily. If we circle a vertex $P \neq O$ in the positive sense, the first alternative follows the unique edge at P which is not an alternative. Those vertices which possess alternatives will be called *moves;* the remaining vertices will be called *plays*. We define a partition[3] of the moves into sets A_j, $j = 1, 2, \ldots$, where A_j contains all of the moves with j alternatives, which will be called the *alternative partition.*

We also introduce a *temporal order* in the tree K. As in any tree with base point O, there is a uniquely defined unicursal path W_P leading from O to the vertex P. We say that $P \leqq Q$ whenever $P \in W_Q$. This clearly defines a partial ordering of the vertices of K and enables us to assign a (temporal) *rank* to the vertices as follows: O is of rank 1. A vertex P is of rank k if the maximum of the rank of Q such that $Q < P$ is $k - 1$. Using this definition we can introduce the *rank partition* of the moves into sets M_k consisting of all moves of rank k for $k = 1, 2, \ldots$.)

(2) A partition of the moves into $n + 1$ indexed sets $P_0, P_1, \ldots, P_n$ which will be called the *player partition*.

(*Terminology:* The moves lying in P_0 are called *chance moves;* all other moves are called *personal moves.*)

(3) A partition of the moves into sets U which is a refinement of the alternative, player and rank partitions, that is, each U is contained in $P_i \cap A_j \cap M_k$ for some i, j and k. This partition is called the *information partition* and its sets will be called *information sets.*

(4) For each $U \subset P_0 \cap A_j$, a probability distribution on the integers $1, \ldots, j$, which assigns *positive* probability to each. Such U are assumed to be one-element sets.

(5) An n-tuple of real numbers $h(W) = (h_1(W), \ldots, h_n(W))$ for each play W.

(*Terminology:* The function h specified in (5) is called the *pay-off function.*)

The question which must be answered immediately is: How is this formal scheme to be interpreted? That is, how does one play our general n-person game Γ? To personalize the interpretation, one may imagine a number of people isolated from each other with contact with a single person, termed the *umpire.* All persons involved are supposed to know the rules of the game; that is, each is to have a copy of the tree K and the specifications (1)–(5). We assume that there is one person for each information set and that they are grouped into players in the natural manner, a person belonging to the ith player if his information set lies in P_i. This seeming plethora of persons is occasioned by the possibly complicated state of information of our players who may be called upon to forget facts which they knew earlier in a play.[4]

A play begins at the vertex O. We do not exclude the possibility that this is the only vertex in K; then we have a no-move game, no one does anything and the pay-off is $h(O) = (h_1(O), \ldots, h_n(O))$. Suppose that the play has progressed to the move P. Then the play continues by the umpire contacting the person whose information set contains P and, if P is a personal move with j alternatives, asking him to choose a positive integer not greater than j. The person does this, knowing only that he is choosing an alternative at one of the moves in his information set. We assume that the umpire makes all of the chance choices in advance, in accord with the probabilities assigned in (4), so that if P is a chance move then an alternative has already been chosen. In this manner, a path with initial point O is constructed. It is unicursal and hence leads to a play W. (Henceforth we shall utilize the 1–1 correspondence between the plays, which are vertices, and the unicursal paths from O to the plays and use the name play for both objects when no confusion will result.) At this point, the umpire pays player i the amount $h_i(W)$ for $i = 1, \ldots, n$.

A detailed comparison of our formal scheme and von Neumann's axiomatic formulation[5] reveals that if we derive one of our games from a von Neumann game in a natural way the only condition imposed is that all of the plays be of the same rank. This is essentially trivial and can be satisfied by filling out "short" plays with dummy chance moves with only one alternative. Proceeding in the converse direction, if we take one of our games in which all of the plays have the same rank, we impose the single restriction that all of the plays in Ω be admissible under the rules of Γ. Thus we have axiomatized essentially the same set of objects as von Neumann.

2. Pure, Mixed and Behavior Strategies.—Rather than make each decision separately as the occasion demands, a player may devise a plan in advance to cover all possible situations which may confront him. He loses nothing by doing this since he makes his choice a function of the information available to him; consequently, his choice must be constant over each of his information sets. Such a plan is called a *pure strategy.*

Definition: A *pure strategy* π_i for player i in Γ is a choice of a positive integer not greater than j for each set $U \subset P_i \cap A_j$.

If the players choose pure strategies $\pi_1, \ldots, \pi_n$ then a probability p_e is assigned to each alternative e in the graph K; if e is a chance alternative, then p_e is obtained from specification (4), while if e is the νth alternative at a personal move in P_i then $p_e = 1$ if π_i specifies the choice ν on the set U containing this move and $p_e = 0$ otherwise. Clearly, the probability $p_W(\pi_1, \ldots, \pi_n)$ that a play W will occur is given by the formula:

$$p_W(\pi_1, \ldots, \pi_n) = \prod_{e \in W} p_e(\pi_1, \ldots, \pi_n), \tag{6}$$

and hence the expected pay-off to player i is given by

$$H_i(\pi_1, \ldots, \pi_n) = \sum_W p_W(\pi_1, \ldots, \pi_n) h_i(W). \tag{7}$$

Unfortunately, our definition of a pure strategy, while conceptually simple, has an inherent redundancy which we will now eliminate. This redundancy is simple in nature; in the case of a zero-sum two-person game, it is merely the duplication of rows and columns in the pay-off matrix.

Definition: We shall say that two pure strategies for player i are *equivalent*, written $\pi_i \equiv \pi_i'$, if and only if $p_W(\pi_1, \ldots, \pi_i, \ldots, \pi_n) = p_W(\pi_1, \ldots, \pi_i', \ldots, \pi_n)$ for all plays W and all pure strategies for the remaining players, $\pi_1, \ldots, \pi_{i-1}, \pi_{i+1}, \ldots, \pi_n$.

The following definition provides the working criterion for the equivalence of pure strategies.

Definition: A personal move P for player i is called *possible when playing* π_i if there exists a play W and pure strategies for the remaining players $\pi_1, \ldots, \pi_{i-1}, \pi_{i+1}, \ldots, \pi_n$ such that $p_W(\pi_1, \ldots, \pi_i, \ldots, \pi_n) > 0$ and $P \in W$.

Criterion: The pure strategies π_i and π_i' are equivalent if and only if they have the same set of possible moves and specify the same choices on those moves.

Henceforth, when we speak of a pure strategy, we shall mean an equivalence class under the definition just given. Clearly, formulae (6) and (7) still hold, where if e is an alternative specified by a pure strategy on an information set containing a possible move then $p_e = 1$ and $p_e = 0$ otherwise.

However, the simplest games (e.g., Matching Pennies) reveal that a player is at a disadvantage if he uses the same pure strategy in each play. Instead, he can use a probability distribution μ_i on his pure strategies which we will call a *mixed strategy*. Now let π_i appear in the mixed strategy μ_i with probability q_{π_i}. Then the probability that a given play W will result is:

$$p_W(\mu_1, \ldots, \mu_n) = \sum_{\pi_1, \ldots, \pi_n} q_{\pi_1} \cdots q_{\pi_n} p_W(\pi_1, \ldots, \pi_n) \tag{8}$$

and the expected pay-off to player i is given by

$$\begin{aligned} H_i(\mu_1, \ldots, \mu_n) &= \sum_W p_W(\mu_1, \ldots, \mu_n) h_i(W) \\ &= \sum_{\pi_1, \ldots, \pi_n} q_{\pi_1} \cdots q_{\pi_n} H_i(\pi_1, \ldots, \pi_n). \end{aligned} \tag{9}$$

In solving games in normalized form it has been customary to deal with the mixed strategies just introduced. However, instead of mixing pure strategies, a player could specify a probability distribution over the alternatives in each information set and thus plan his action in any given play. We will call the aggregate of such distributions a *behavior strategy*. The advantage of dealing with behavior strategies is a radical reduction of the dimension of the sets involved while the obvious disadvantage derives from a loss of freedom of action. Behavior strategies have been used with telling effect in the solution of individual games by von Neumann,[6] J. Nash and L. S. Shapley,[7] and the author.[8] In our formal treatment we will only deal with behavior strategies which are derived from a mixed strategy.

Definition: Suppose that the mixed strategy μ_i assigns the probability q_{π_i} to each pure strategy π_i and consider the information set U for player i with j alternatives. Let S be the set of pure strategies π_i such that some $P \in U$ is possible when playing π_i. Then S is the disjoint union of the sets $S_1, \ldots, S_j$ where S_ν consists of all π_i which specify the νth alternative on U. If $\sum_{\pi_i \in S} q_{\pi_i} \neq 0$ then we define:

$$b_\nu = \sum_{\pi_i \in S_\nu} q_{\pi_i} \Big/ \sum_{\pi_i \in S} q_{\pi_i} \quad \text{for } \nu = 1, \ldots, j. \tag{10}$$

If $\sum_{\pi_i \in S} q_{\pi_i} = 0$ then no distribution is defined. The aggregate of all such assignments on information sets U for player i is called the *behavior strategy associated with* μ_i and is denoted by $\beta_i(\mu_i)$ or simply β_i.

Again, if the players choose behavior strategies $\beta_1, \ldots, \beta_n$ then a probability p_e is assigned to each alternative e in the graph K; if e is a chance alternative, then p_e is obtained from specification (4), while if e is the νth alternative at a personal move in P_i at which β_i assigns the probability b_ν then $p_e = b_\nu$ and $p_e = 0$ otherwise. Clearly, the probability $p_W(\beta_1, \ldots, \beta_n)$ that a play W will occur is given by the formula:

$$p_W(\beta_1, \ldots, \beta_n) = \prod_{e \in W} p_e(\beta_1, \ldots, \beta_n) \tag{11}$$

and hence the expected pay-off to player i is given by

$$H_i(\beta_1, \ldots, \beta_n) = \sum_W p_W(\beta_1, \ldots, \beta_n) h_i(W). \tag{12}$$

3. Games with Perfect Information and Games with Perfect Recall.— We shall be concerned with two large classes of games in which the information partition assumes a special form.

Definition: A game Γ is said to have *perfect information*[9] if the information partition consists of one-element sets.

Definition: A game Γ is said to have *perfect recall for player* i if, for all pairs of moves P, Q for player i such that $P < Q$, we have the following condition satisfied. Assume that P and Q lie in the information sets U and V, respectively. Let P have j alternatives and let V_ν be the set of all moves following some $R \in U$ in the temporal order in a play which has the νth alternative at R. Then we demand that $V \subset V_\nu$ for some ν. A game Γ is said to have *perfect recall* if it has such for all players.

The interpretation of these terms is exactly what the names imply. In a game with perfect information, each player is informed at every move of the exact sequence of choices preceding that move. In a game with perfect recall each player remembers everything that he knew and all of his choices at previous moves. The following two theorems hold for such games.

THEOREM 1. *A sufficient condition that an n-person game* Γ *have an equilibrium point*[10] *among the pure strategies for all possible assignments of the pay-off function* h *is that* Γ *have perfect information.*

THEOREM 2. *A necessary and sufficient condition that*

$$H_i(\beta_1(\mu_1), \ldots, \beta_n(\mu_n)) = H_i(\mu_1, \ldots, \mu_n) \tag{13}$$

for all mixed strategies $\mu_1, \ldots, \mu_n$ *and* $i = 1, \ldots, n$ *in an n-person game* Γ *for all possible assignments of the pay-off function h is that* Γ *have total recall.*

Theorem 1 generalizes the theorem of von Neumann which asserts that a zero-sum two-person game with perfect information is strictly determined. It is proved by the same inductive device with a slight variation due to the absence of the minorant and majorant games in the general n-person case. Theorem 2 enables us to replace mixed strategies by behavior strategies in games with total recall and has many computational ramifications. The proofs of both of the theorems and further considerations of extensive games will be published elsewhere.

* Written under a contract with the Office of Naval Research.

[1] Neumann, J. von, and Morgenstern, O., *The Theory of Games and Economic Behavior*, 2nd ed., Princeton University Press, 1947.

[2] A graphical representation by a tree has been suggested by von Neumann, *loc. cit.*, p. 77, however he does not treat this matter systematically, preferring a set theoretical formulation.

[3] In this paper a partition means an exhaustive decomposition into (possibly void) disjoint sets.

[4] It has been noted by von Neumann that Bridge is a two-player game in exactly this manner.

[5] Neumann, J. von, and Morgenstern, O., *loc. cit.*, pp. 67–84.

[6] Neumann, J. von, and Morgenstern, O., *loc. cit.*, pp. 192–194.

[7] Nash, J., and Shapley, L., "A Simple Three-Person Poker Game," *Annals of Mathematics*, Study No. 24 (in preparation).

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THE SPECIFICITY OF ANTI-KIDNEY ANTIBODY DETERMINED BY ITS EFFECT UPON TISSUE CULTURE EXPLANTS

BY RICHARD W. LIPPMAN, GLADYS CAMERON AND DAN H. CAMPBELL

INSTITUTE FOR MEDICAL RESEARCH, CEDARS OF LEBANON HOSPITAL, LOS ANGELES, CALIFORNIA, AND THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY,* CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA†

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While investigating the pathogenesis of experimental nephritis produced by rabbit anti-rat-kidney antibody, it occurred to us that the effects of anti-kidney antibody on kidney tissue might readily be visualized in tissue cultures. The specificity of tissue antigens has previously been investigated by the usual immunologic procedures[1] and the effects of antibodies[2, 3] upon tissue explants has long been known. The growth and function of tissue explants have previously been used to study the specificity of tissue

antibodies,[4, 5] but the antisera used were of low, uncertain potency, and only a few different tissues were examined in testing the toxic effects. The following study shows that rabbit anti-rat-kidney gamma globulin is toxic for tissue explants of brain and heart muscle, as well as kidney. These effects are of low species specificity and may be observed in cultures prepared from chick embryo tissues, as well as those prepared from rat tissues. In addition, the serum of patients with glomerular nephritis was found to contain a substance with similar effects and specificity.

Methods.—Rabbit anti-rat-kidney serum was prepared by immunizing rabbits over a period of 4 months with a suspension of rat kidney tissue. The serum was first adsorbed with a suspension of sheep erythrocytes, to remove the Forssman antibodies, and then with rat erythrocytes. The antibody globulin was precipitated by $^1/_2$ saturation with ammonium sulfate at pH 7.8. This preparation (NTG) was found by the Tiselius pattern to be composed entirely of gamma globulin. The minimal serological activity was determined by a precipitin test with the soluble portion of the original antigen and the preparation NTG was found to contain at least 12% precipitable antibody.

Tissue explants were cultured by the usual double cover slip method, which has been described in detail.[6] In a five-drop portion of culture medium, one drop of NTG (32.5 mg. protein/ml.) was substituted for one drop of serum. Controls were concurrently grown in a medium that substituted one drop of gamma globulin prepared from unimmunized rabbit serum (GC) for one drop of serum. Controls were also grown, in most cases, in a medium of the usual formula without substitution (SC).

Cultures were examined at intervals up to 72 hours, when the experiments were usually terminated, and were graded for growth and evidence of functional activity. At least 4 explants were grown under each condition, and the key conditions were repeated for several groups of 4 explants each. The results were completely uniform. Photographs were taken of typical preparations for permanent record, and some of the tissues were then fixed and stained.

Species Specificity.—Explants of rat kidney in SC and GC grew very well. Those in NTG showed virtually no growth, and in the few cells that grew out from the explant the cytoplasm was granular, with fatty degeneration. Most of these cells were dying, and many became autolyzed during the period of observation.

In order to provide a crucial test of species specificity, explants of chick mesonephros were grown in rat NTG. Almost no growth occurred. In addition, tubular function in the explant, easily visualized by the incorporation of a low concentration of phenol red in the medium,[7] rapidly came to a halt. In contrast, excellent growth and function were observed in SC and GC preparations.

Organ Specificity.—In order to study organ specificity of NTG, tissue explants from different organs of the rat and of the chick embryo were grown in the various media. The results are shown in table 1. As seen from the table, growth of kidney, heart and brain explants was inhibited by NTG. No other tissues were affected, although a wide variety derived from all embryonic layers was examined.

Circulating Nephrotoxin in Patients with Glomerular Nephritis.—It has been well known, since the work of Masugi,[8] that a form of nephritis may

TABLE 1

EFFECT OF RABBIT ANTI-RAT-KIDNEY GLOBULIN ON TISSUE EXPLANTS FROM THE CHICK EMBRYO AND FROM THE RAT

SPECIES	TISSUE	SERUM CONTROL (SC)	GLOBULIN CONTROL (GC)	NEPHROTOXIC GLOBULIN (NTG)
Rat	Kidney	0	0	+
	Heart	0	0	+
	Brain	0	0	+
	Liver	*	0	0
	Bladder	*	0	0
	Spleen	*	0	0
Chick	Mesonephros	0	0	+
	Heart	0	0	+
	Brain	0	0	+
	Liver	*	0	0
	Striated muscle	0	0	0
	Lung	0	0	0
	Intestinal smooth muscle	0	0	0
	Intestinal mucosa	0	0	0
	Skin	0	0	0
	Tongue	0	0	0
	Chorio-allantoic membrane	*	0	0

+ = marked inhibition of growth, 0 = no effect on growth.
* No experiment performed.

be produced in animals by the administration of anti-kidney serum. Lange and his coworkers have reported the occurrence of "auto-antibodies" to kidney tissue in human nephritis.[9] For these reasons, it seemed desirable to test the serum of patients with glomerular nephritis, but without renal failure, for the presence of a circulating nephrotoxin. The serum of 6 patients and 4 normal individuals has been tested in this way, and the results are presented in table 2. Serum from all of the patients with well-documented glomerular nephritis completely inhibited the growth and tubular function of explants from the chick mesonephros. Growth of chick heart

and brain explants was also inhibited. Serum from normal individuals had no effect upon the explants when compared with controls grown in SC.

Comments.—It is of considerable interest that rabbit anti-rat-kidney gamma globulin inhibits the growth in tissue culture of rat heart muscle as well as rat kidney, chick brain, chick heart muscle and chick mesonephros. The presence of a common antigen in these tissues would render reasonable the clinical association of such conditions as glomerular nephritis, rheumatic carditis and chorea.

Even more interesting is the observation that the serum of patients with glomerular nephritis contains a substance which is directly toxic to kidney, heart and brain tissue. Since the patients were selected for adequate renal function, the effect probably cannot be attributed to accumulated waste metabolites in the serum.

TABLE 2

EFFECT ON GROWTH OF CHICK EMBRYO TISSUES OF SERA FROM NORMAL INDIVIDUALS AND FROM PATIENTS WITH GLOMERULAR NEPHRITIS

SUBJECT	DIAGNOSIS	STAGE[10]	MESO-NEPHROS	HEART	BRAIN	STRIATED MUSCLE
R. L.	Normal	...	0	0	0	0
H. U.	Normal	...	0	0	0	0
H. Y.	Normal	...	0	0	0	0
A. M.	Normal	...	0	0	0	0
B. M.	Glom. neph.	Initial	+	+	+	0
A. R.	Glom. neph.	Degenerative	+	+	+	0
K. R.	Glom. neph.	Degenerative	+	+	+	0
D. W.	Glom. neph.	Degenerative	+	+	+	0
C. B.	Glom. neph.	Degenerative	+	+	+	0
W. R.	Glom. neph.	Degenerative	+	+	+	0

+ = marked inhibition of growth, 0 = no effect on growth.

Further experiments to advance all phases of this study are in progress. It is hoped that we shall be able to characterize more completely the organ and species specificity of several tissue antigens and that we shall be able to study in more detail the localization of antibody activity in the various serum protein fractions. Sera from the patients will be fractionated in order to identify the nephrotoxin. It is also hoped that these studies will lead to a simple test for the presence of tissue toxins, and an effort will be made to neutralize such a substance *in vivo*.

The technique of tissue culture offers a sensitive and relatively simple way to examine sera for the presence of tissue toxins and antibodies. It is our ultimate intention to extend these studies to patients with other diseases thought to be related to tissue allergies, such as lupus erythematosus disseminatus, rheumatic fever, rheumatoid arthritis and others.

* Contribution No. 1442.

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*THE EXCITATORY PROCESS IN THE COCHLEA**

BY H. DAVIS, C. FERNÁNDEZ,† AND D. R. MCAULIFFE

CENTRAL INSTITUTE FOR THE DEAF, ST. LOUIS, MISSOURI

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Introduction.—The nature of the excitatory process by which sensory cells initiate nerve impulses in afferent nerve fibers is very obscure. In the case of the ear, the most widely accepted theory has been that an electrical potential known as the "cochlear microphonic" is generated by the hair cells in the organ of Corti and serves as a direct electrical stimulus to the peripheral terminations of the fibers of the auditory nerve.

The cochlear microphonic, it will be recalled, seems to be simultaneous with the mechanical movements of the cochlear partition and it follows the wave form of the sound very closely, at least at moderate intensities of stimulation. It shows no refractory period or all-or-none characteristics like the action potentials of nerve.[1] Action potentials of the auditory nerve, probably generated in the cell bodies in the spiral ganglion, not in the fine non-myelinated terminal twigs, can also be recorded from electrodes placed in or near the cochlea.

Sound waves at 2000 to 6000 cycles per second are very efficient as auditory stimuli. This high efficiency led Wever[1] and one of the present authors[2] to postulate an intermediate excitatory effect or process which had the property of summating the excitatory effects of two or more sound waves. We now present experimental evidence for the existence of such a process of summation. We also have found a third electrical potential in the cochlea in addition to the cochlear microphonic and nerve action potentials. The new potential exhibits summation and seems to represent the local excitatory process that initiates auditory nerve impulses.

Methods.—All experiments were performed on guinea pigs anesthetized with dial in urethane. Electrodes consisting of No. 38 enamel-insulated silver wire were introduced into the cochlea through small holes drilled into scala tympani, scala vestibuli and/or scala media[3, 4] of the basal turn.

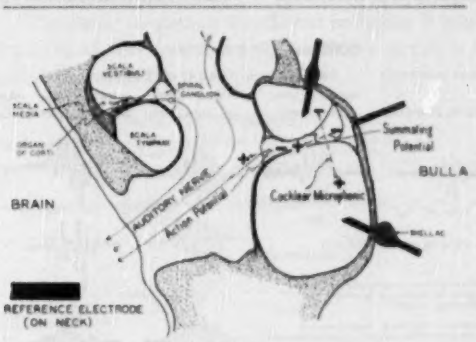

FIGURE 1

(In some experiments electrodes were placed in other turns as well.) The position in turn 1 is at the site of maximal stimulation by tones of 8000 c. p. s. The electrical circuit was completed by a reference electrode attached to the wound in the neck. The cochlea of the guinea pig protrudes into an air-filled bulla, and thus the electrical circuit from the reference electrode enters the cochlea chiefly by way of the internal auditory meatus. The reference electrode is thus roughly equivalent to one placed on the auditory nerve.

Electrical responses were recorded simultaneously from several combinations of electrodes by means of a three-channel cathode-ray oscilloscope assembly (Grass). Two of the channels could be connected in parallel so as to either add or subtract the signal in one to (or from) the signal in the other.[3, 4] In this way the action potential of the auditory nerve, which appears as a negative electrical change at all of the cochlear electrodes,

could be completely canceled by subtracting the scala tympani signal from the scala vestibuli signal, after appropriate adjustment of amplification. The cochlear microphonic, on the other hand, appears in opposite phase in scala tympani from what it is in scala vestibuli and scala media (see Fig. 1). It can be canceled by adding the signals from scala vestibuli (or media) and scala tympani. By this method action potentials of moderate voltage can be viewed, and their latency measured, in spite of the simultaneous presence of a large cochlear microphonic which otherwise usually obscures them almost completely.

The acoustic stimuli employed included clicks and pure tones, but were chiefly brief tone-pips. Our usual "tone-pip" consisted of sound waves at a basic frequency of 8000 c. p. s. This signal begins gradually and reaches maximum amplitude during the fourth or fifth wave and immediately diminishes again (see Fig. 3). The "2000 c. p. s. tone-pip" has a basic frequency of 2000 c. p. s. The maximum in this case is reached

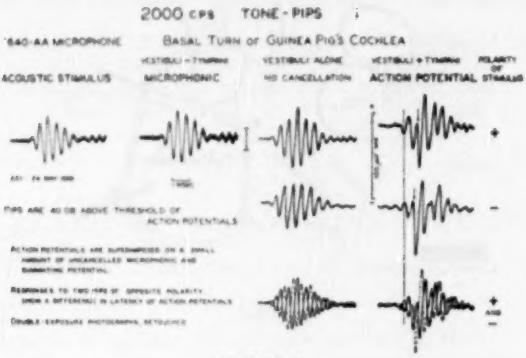

FIGURE 2

during the third sound wave. The pips were presented at pulsing frequencies from 1 to 60 per second. The tone-pips have several advantages: (1) nearly all of the acoustic energy is concentrated in a band less than an octave wide[5] so that they presumably activate a relatively restricted region of the cochlear partition, yet (2) the relatively rapid onset may allow identification of the particular sound wave that initiates a nerve impulse and (3) at frequencies of 1000 c. p. s. and higher only one impulse is set up in each fiber because of the rapid increment and decrement of sound waves and the refractory period of the nerve fibers.

Evidence for Summation.—For frequencies of 2000 c. p. s. and less, each volley of nerve impulses revealed by the action potential can be clearly associated with one sound wave or another. We find that only the portion

of the wave corresponding to acoustic rarefaction at the ear drum causes auditory stimulation. If one (negative) wave in a tone-pip has set up an action potential, the next wave, if stronger, may activate other neurons of higher threshold even though the neurons stimulated by the first wave are still refractory. Thus the composite action potential may show two peaks separated by one wave-length of the basic frequency of the tone-pip (see Fig. 2). Reversal of the polarity of the tone-pip, i.e., starting with a negative instead of a positive sound wave, will change the latency of the first effective sound wave because the first adequate negative sound wave now comes either a half wave earlier or a half wave later than it did previously. (The first effective wave may be either stronger or weaker than the first effective wave with the original polarity. The two peaks of action potentials will in general, therefore, be different in amplitude as well as showing a difference in latency. Both of these effects are illustrated in figure 2.) The basic frequency employed in figure 2 was 2000 c. p. s. and it is clear that at this frequency the auditory nerve is responding to individual sound waves in the tone-pip.

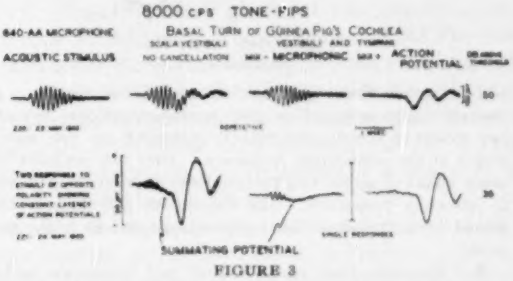

FIGURE 3

With a basic frequency of 8000 c. p. s., however, the behavior of the nerve is fundamentally different. There is no visible change in latency when the polarity of the tone-pip is reversed (see Fig. 3). We have sought for a change of latency with great care on the face of the oscilloscope and have assured ourselves that if there is a change of latency it is certainly less than 30 microseconds. The change which should occur if the nerve were responding to individual sound waves is 62 microseconds. We conclude that at this frequency the nerve does not respond to individual sound waves but that a stimulating effect is carried over, even from the earliest subliminal waves. The stimulating effects are integrated by some process that we call "summation" so that the nerve responds to the tone-pip as a whole instead of to the individual waves. Summation seems to dominate the picture at 8000 c. p. s. (At 4000 c. p. s. a small change of latency with reversal of polarity can still be demonstrated, however.)

A second line of evidence for summation at high frequencies is found in the change of latency with the intensity of a tone-pip. At low frequencies the latency diminishes in stepwise fashion as the intensity of the stimulus is increased. Each step means that an earlier sound wave, originally too weak to stimulate, has now become strong enough to set up nerve impulses. At 2000 c. p. s. the number of steps that can be observed corresponds to the number of sound waves (counting only the negative peaks) from the beginning of the tone-pip to the largest negative peak. At 8000

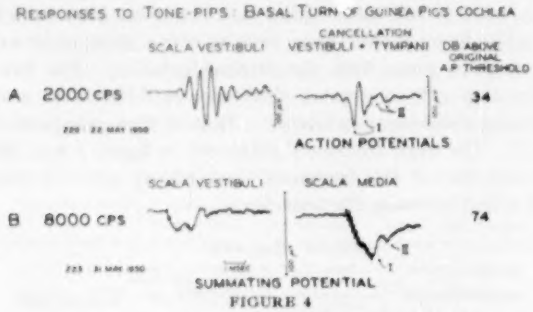

FIGURE 4

A. Cancellation of cochlear microphonic of 2000 c. p. s. tone-pip (gains adjusted to give minimum residue) reveals two volleys of action potentials (I) separated by one wave-length of the stimulating frequency. They are preceded by small waves of summating potential and cochlear microphonic in uncertain proportion. The late waves (II) include the second neural response (from the cochlear nucleus in the medulla).

B. Responses from scala vestibuli and from scala media (slightly retouched). No cancellation. In this experiment the cochlear microphonic disappeared almost completely when an electrode was inserted into scala media. Small action potentials are visible and the 8000 c. p. s. ripple is partly cochlear microphonic but the main deflections are summating potential. An upward deflection means cochlea more positive to reference electrode.

c. p. s., however, the shortening of latency as intensity increases seems to be continuous. More important, the total shortening as the stimulus is increased from threshold to a rather high level may be greater by at least two wave-lengths than the time from the beginning of the pip to the largest wave. It seems, therefore, that at threshold the process of stimulation must have been completed by a wave later than, and therefore smaller than, the maximum wave at the center of the tone-pip. We believe that this is good supporting evidence for the existence of summation, although

the argument makes the tacit but unproved assumptions that the conduction time for the nerve impulse and also the time required for the excitatory process to set off a nerve impulse are the same whether the stimulus be weak or strong.

The Summating Potential.—About a year ago[6] we described what we called "rectification" of the cochlear microphonic. Under several adverse circumstances, such as anoxia, the electrocoagulation of part of the cochlea, or the insertion of several electrodes deeply into the cochlea, the cochlear microphonic may apparently shrink to a few per cent of its original amplitude. The remaining response is "rectified," meaning that only one-half of each wave (the half in which scala vestibuli becomes electrically negative) still remains. This description is adequate for frequencies below 2000 c. p. s. We have recently observed that at higher frequencies there is also a shift of the base line in the direction of negativity in the scala vestibuli. The negative deflection appears to be produced by fusion of successive electrical pulses that recur with the frequency of sound waves. With an 8000 c. p. s. tone-pip the pattern resembles the negative half of the envelope of the pip, with an 8000 c. p. s. ripple also clearly visible (see Fig. 4). These "rectified" potentials are actually quite independent of the cochlear microphonic. They represent a third electrical potential of the cochlea which shows the property of summation at frequencies above 2000 c. p. s. This third potential decays rapidly enough, however, so that there is usually no effective carry-over (summation) below 2000 c. p. s. The maximum voltage of the third potential has not yet been measured because of serious technical limitations but it is well over 50 microvolts (scala media vs. reference electrode).

Evidence that the summating potential differs from both the cochlear microphonic and the action potential is as follows:

1. The summating potential (SP) is unidirectional (like the action potential) for any particular pair of electrodes. The cochlear microphonic (CM), on the other hand, is a change of electrical potential both above and below the original resting level.

2. SP shows summation. It outlasts the mechanical movement (unlike CM) and it shows no all-or-none behavior or refractory period (unlike action potentials (AP)).

3. SP and CM are differently oriented anatomically. The most favorable combination of electrodes for recording SP is scala media vs reference electrode. The most favorable combination for CM is scala vestibuli vs. scala tympani. Figure 1 shows how these axes are approximately at right angles to one another.

4. The anatomical location of SP is apparently in the organ of Corti (like CM) and not in the spiral ganglion (unlike AP). AP appears equally and in the same direction at electrodes in scala vestibuli, media or tympani

(vs. reference electrode). SP appears strongly in scala media (vs. reference) but may be completely absent in scala tympani (unlike CM and AP), or, if the electrode is placed as far as possible away from the basilar membrance, its sign may actually be reversed. The possibility of placing an electrode on the positive side of the isopotential line in scala tympani shows that the origin of the potential difference must be well out in the cochlear canal and not within the modiolus.

5. The SP lags in time (or phase) behind CM. If CM from scala tympani is used to cancel CM from scala vestibuli, some SP as well as AP therefore remains (see Fig. 4 *A*). The lag seems to be of the order of 100 microseconds.

6. The SP may remain in the face of adverse conditions, such as anoxia or operative trauma, after the cochlear microphonic has virtually or completely disappeared. Action potentials have been observed under such conditions of operative trauma. This point is of special importance as it shows that *neither the summating potential nor the action potentials are in any way dependent on the cochlear microphonic.* The cochlear microphonic may be a good indicator of the time and a fair indicator of the intensity of mechanical movement of the organ of Corti, but it is not part of the chain of events that generates the nerve impulse.

7. At high intensities of stimulation, although still within limits perfectly tolerable to the human ear, the SP continues to increase with the intensity of the 8000 c. p. s. tone-pips, although the increase (like that of CM) becomes non-linear. While SP is still increasing CM reaches its maximum and may even begin to shrink again. It is worth noting that the behavior of SP is more in line with the behavior of AP, which is still increasing at this intensity, not to mention the sense of loudness to the human ear which is also still increasing.

8. Electrical polarization between scala vestibuli and scala tympani increases or decreases SP according to its direction. When the SP is increased the AP increases also. When SP is diminished AP diminishes or vanishes. The same intensity of electrical polarization causes a small, but only very small, change in the cochlear microphonic. If the cochlear microphonic is already absent it may be restored (or an equivalent microphonic may be introduced) by electrical polarization. The polarity of the microphonic relative to the sound wave will in this case depend on the direction of the polarizing current. We have not been able to reverse SP by polarization.

9. On the death of the animal AP disappears first; SP continues for a time after death; CM may continue longer, although at the low post-mortem level that is already so familiar.[7] Sometimes SP outlasts CM.

Interpretation of the Summating Potential.—We believe that the summating potential arises in the terminal twigs of the auditory nerve fibers

which are arranged like baskets around the hairless ends of the hair cells. We believe also that the summating potential is a local excitatory process analogous to the end-plate potential at the neuro-myal junction and to the post-synaptic potentials of spinal neurons.[8] How the mechanical movement sets up the local excitatory process (the summating potential) is unknown.

The latency of the action potential relative to the mechanical movement we interpret as due mostly to conduction time from the nerve endings to the cell bodies. The shortest latency that we have measured for the foot of the action potential from the beginning of the cochlear microphonic following a strong, sharp click (rarefaction) is 550 microseconds.

General Significance.—We have described a summating potential which seems to represent the excitatory process initiated by the sensory cells of the cochlea in their afferent nerve fibers. Similar electrical potentials, probably representing the same type of excitatory process, have been recorded from muscle spindles[9] and from Pacinian corpuscles,[10] and by analogy we may expect to find them also in the related sense organs for touch, pressure, vibration, muscle sense, orientation to gravity and acceleration. The summating potential in the ear and perhaps elsewhere should be a useful tool for determining the mode of action of drugs, fatigue, etc., on the sense organ and for investigation of the process whereby mechanical force initiates the excitatory process.

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† Former Fellow of the W. K. Kellogg Foundation.

‡ Read under the titles: "Audition—A Physiological Survey" and "Summation in the Auditory Sensory Process." Some material has been added in preparing the paper for the press.

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SEXUAL DIFFERENTIATION IN RHABDOSTYLA VERNALIS*

By HAROLD E. FINLEY AND PHILIP A. NICHOLAS

DEPARTMENT OF ZOOLOGY, HOWARD UNIVERSITY

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Introduction.—The problem of sex in the protozoa has claimed the attention of many investigators. The most fruitful explorations of the problem stem from the studies on Paramecium made by Sonneborn,[1] Jennings,[2] and their colleagues. Working in a similar subject matter area, but with a different protozoan, Finley[3] ascertained the origin of conjugating individuals of *Vorticella microstoma* and reported evidence regarding their sexual differentiation. In *V. microstoma* conjugation was found to be a sexual type of reproduction which involved the union of a micro- and macroconjugant, the two organisms becoming one entity and offspring being produced therefrom. A preconjugation fission was described and interpreted as the conjugant (sex ?) differentiating fission.

This investigation was undertaken to extend the knowledge of conjugation in peritrichous protozoa. It was believed that new findings would be of considerable importance in understanding sexual differentiation in protozoa and might lead to contributions properly bearing upon broad problems of sex.

Rhabdostyla vernalis was investigated by methods similar to those previously reported by Finley.[3] Results obtained thereby indicate that sexual differentiation in Rhabdostyla is comparable but not identical to the phenomenon discovered in Vorticella.

Experimental.—A parental stock of Rhabdostyla was established by allowing a single individual to multiply vegetatively. Its progeny were used throughout the course of this study. The organisms were cultivated in Columbia culture dishes from which other kinds of protozoa were meticulously excluded. The cultures were stored in covered moisture chambers. Subcultures from the parental stock were made by transferring approximately twenty-five telotrochs to a sterile dish containing sterile culture medium. This method of establishing subcultures made it unnecessary to inoculate the new medium with bacteria (which served as food for Rhabdostyla) since adequate numbers were carried along with the telotrochs.

Occasionally encysted specimens were obtained by the well-known method of sealing the culture vessel with a glass cover on which petrolatum had been smeared in order to exclude air. Encysted specimens were induced to excyst then allowed to multiply until their progeny entered into an epidemic of conjugation. However, the most reliable method for inducing conjugation was the following one: Old cultures containing sparse

animated populations and a few encysted Rhabdostyla (in a state of suspended animation) were activated by decanting the old culture medium and replacing it with three changes of distilled water; the water was replaced by a sterile culture medium consisting of equal parts of 1% aqueous solution of cerophyl,[4] filtered casein broth containing salts customarily included in Knopf's solution, and distilled water. Successful activation of the cultures resulted in a series of rapid vegetative fissions which continued for approximately 12 to 36 hours and culminated in the unique preconjugation fissions. Preconjugation fissions yielded macro- and microconjugants.

Live specimens were studied with the aid of widefield microscopes and also compound research type microscopes. The usual methyl-green-acetic and Feulgen and Heidenhain's preparations were studied. The abundant supply of material at hand made it possible to observe isolated specimens or mass cultures in the various stages of reproduction. Fortunately, this was done almost at the will of the observers.

Observations.—In the main, nuclear and cytoplasmic phenomena of *R. vernalis* were similar to those reported for *V. microstoma*. During vegetative and preconjugation fissions the macronucleus underwent a nonmitotic division. The micronucleus divided by mitosis. Each daughter product of vegetative fission received, approximately, an equal volume of the macronucleus and the cytosome. Each microconjugant got only $^1/_2$ as much macronuclear and cytosomal material as its sister macroconjugant. The micronuclear material seemed to be equally distributed during the progress of preconjugation and vegetative fissions.

There was no apparent morphological difference between rhabdostylas about to undergo vegetative fission and those on the verge of preconjugation fission. Morphological differences between macro- and microconjugants appeared only when the preconjugation fission was well underway.

Each "neuter Rhabdostyla" usually produced one macroconjugant and four microconjugants, in contrast with one of each from *V. microstoma*. That difference can be explained. Rhabdostyla's microconjugant remained attached to its sister macroconjugant at the conclusion of unequal (preconjugation) fission and after a lapse of 5 to 10 minutes it began a fission which yielded two microconjugants of equal size. These two specimens, while still attached to the macroconjugant, divided by equal fission, the result being four microconjugants attached near the basal region of the macroconjugant. All four microconjugants developed a posterior ciliary wreath, detached themselves one after another and swam away.

The fate of microconjugants was determined to be either conjugation or death. Macroconjugants had a different fate. They were attractive to

microconjugants about two hours; if they were not conjugated within that period they lost their attractiveness and vegetative fission ensued.

We were unsuccessful in our attempts to induce macroconjugants to form protective cysts. Neither have we discovered evidence of autogamy. This strongly suggests that under ordinary circumstances their fate is either conjugation or vegetative fission. In either case, they possess a great progeny potential. When a successful union was established between macro- and microconjugant the usual vorticellid type of conjugation occurred. As previously indicated, an epidemic of conjugation may be imposed upon the progeny of vegetative forms.

The organisms produced by preconjugation fission were the only ones which permanently fused and conjugated. It was relatively easy to prove this fact by isolation experiments. When microconjugants were given ample opportunities to attach themselves to vegetative individuals they failed to do so, even though death was their only apparent alternative.

Summary.—Rhabdostyla's micro- and macroconjugant are derived from a "neuter individual" through an equal division of the micronucleus accompanied by an unequal division of the macronucleus and cytosome. The two products of this fission have different immediate fates although both are potentially destined for conjugation. The larger product is capable of participating in fruitful conjugation without further differentiation. In this respect *R. vernalis* and *V. microstoma* are alike. The smaller product of preconjugation fission is irreversibly destined to produce microconjugants, this being accomplished by two fissions which occur consecutively. Rhabdostyla's microconjugant, unlike the microconjugant of *V. microstoma*, does not seem to be completely differentiated at the same time as its sister macroconjugant.

The situation wherein one undifferentiated microconjugant produces four functional microconjugants is strikingly reminiscent although obviously not identical to the case where a metazoan's primary spermatocyte divides to form two secondary spermatocytes, and they in turn divide to form four spermatids which become functional spermatozoa.

This preliminary résumé of our results will be followed by a more detailed account to be published elsewhere.

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[4] Cerophyl is manufactured by Cerophyl Laboratories, Inc., Kansas City, Mo.

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